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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE
BEFORE THE BOARD OF PATENT APPEALS AND INTERFERENCES

Applicants:	David A. Cheresh et al.	)	
Application No.	09/538,248	)	
Filed:	March 29, 2000	)	Group Art Unit: 1652
For:	METHODS USEFUL FOR TREATING VASCULAR LEAKAGE AND EDEMA USING SRC OR YES TYROSINE KINASE INHIBITORS	)	
Examiner:	Rebecca E. Prouty	)	Attorney Docket No. TSRI 651.3

BRIEF ON APPEAL

Mail Stop Appeal Brief - Patents
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

~~Adjustment date: 01/18/2006 BABRAHA1~~
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~~01 FC:1402 -320.00 OP~~

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01 FC:1402 500.00 OP

Sir:

This is an appeal from the final rejection of claims 1-4, 17-20, 32, and 33 in the above-identified application. This Brief is accompanied by a check in the amount of $180.00 to cover the fee required under 37 C.F.R. §41.20(b)(2), which amount represents the difference between the $500 fee under 37 C.F.R. §41.20(b)(2) and the $320 fee previously paid by Applicants for filing of an Appeal Brief in a prior appeal in this case, in which prosecution was reopened before the appeal went to the Board. Please charge any additional fees concerning this matter or credit any overpayment to our Deposit Account No. 15-0508.

1. Real Party in Interest.

This application is assigned to The Scripps Research Institute, licensed to Merck KGaA, Darmstadt, Germany, and sublicensed to Targegen, Inc.

2. Related Appeals and Interferences.

A Notice of Appeal was filed in this case on April 2, 2003. Prosecution was reopened by the Examiner after Applicants filed their Appeal Brief. A Notice of Appeal was also filed on February 9, 2005 in this case; however, prosecution was reopened when Applicants filed an RCE. Related U.S. Serial No. 10/298,377, which claims priority from the

present application, is also on appeal. To date, no decision from the Board has been received in that appeal. A Notice of Appeal was also filed on September 5, 2002 in related Serial No. 09/470,881 (now US 6,685,938) from which the present application claims priority. Prosecution was reopened upon filing of an RCE and an amendment in response to the final Office Action. The amended claims issued in that case.

3. Status of All Claims.

Claims 1-4, 17-20, 32, and 33 are under appeal. The claims are presented in Appendix A hereto. Claims 1 and 17 are independent claims. Claims 5-16 and 21-31 have been cancelled.

4. Status of All Amendments Filed Subsequent to Final Rejection.

No claim amendments were filed subsequent to final rejection.

5. Concise Summary of the Invention.

The present invention provides methods and articles of manufacture suitable for inhibiting vascular permeability increases in diseased, inflamed or traumatized tissue. Tissue damage related to associated vascular leakage and edema is ameliorated. Vascular permeability is often associated with increased levels of vascular endothelial growth factor (VEGF) in a tissue, and can lead to swelling of and damage to the affected tissue. VEGF is a regulator of both vascular permeability (i.e., the degree of plasma fluid leakage through the blood vessel wall) and of blood vessel formation (i.e., angiogenesis). Surprisingly, the methods and articles of manufacture of the present invention selectively inhibit VEGF-induced vascular permeability without adversely affecting other VEGF-induced responses that can be beneficial to recovery from injury, such as angiogenesis.

Claims 1 and 17 are independent claims. Claims 2-4 and 33 depend either directly or indirectly on claim 1. Claims 18-20 and 32 depend either directly or indirectly on claim 17.

Claim 1 is directed to a method for ameliorating tissue damage related to vascular leakage or edema (pg. 4, lines 17-18; pg. 12, line 14 through pg. 13, line 23) comprising contacting the tissue with a vascular permeability modulating amount (pg. 4, lines 18-20; pg. 35, line 27 through pg. 36, line 21) of a pharmaceutical composition comprising a

human c-Src tyrosine kinase inhibitor (pg. 16, lines 4-5; Figs. 3 and 4; pg. 61, lines 11-19; pg. 62, lines 1-21).

Claim 2 is dependent on claim 1 and specifies that the human c-Src tyrosine kinase inhibitor is a chemical inhibitor (pg. 5, line 21-25).

Claim 3 is dependent on claim 2 and specifies that the chemical inhibitor is selected from the group consisting of pyrazolopyrimidine PP1, pyrazolopyrimidine PP2, PD173955, PD162531, Radicol R2146 and Geldanamycin (pg. 5, lines 21-25; pg. 33, lines 14-16).

Claim 4 is dependent on claim 3 and specifies that the inhibitor is pyrazolopyrimidine PP1 (pg. 5, line 22-23; pg. 33, lines 17-19; pg. 61, lines 11-19; pg. 62, lines 1-21).

Claim 33 is dependent on claim 3 and specifies that the inhibitor is pyrazolopyrimidine PP2 (pg. 5, lines 22-23; pg. 33, lines 19-23).

Claim 17 is directed to an article of manufacture comprising packaging material (pg. 7, lines 12-13; pg. 47, lines 2-20) and a pharmaceutical composition contained within said packaging material (pg. 7, lines 13-14), wherein said pharmaceutical composition is capable of modulating vascular permeability increase in a tissue suffering from a disease condition (pg. 7, lines 14-17), wherein said packaging material comprises a label, which indicates that said pharmaceutical composition can be used for treatment of vascular leakage or edema associated disease conditions (pg. 7, lines 17-20; pg. 47, lines 2-7), and wherein said pharmaceutical composition comprises a human c-Src tyrosine kinase inhibitor (pg. 16, lines 4-5; Figs. 3 and 4) and a pharmaceutically acceptable carrier therefor (pg. 4, lines 20-21).

Claim 18 is dependent on claim 17 and specifies that the human c-Src tyrosine kinase inhibitor is a chemical inhibitor (pg. 5, lines 21-22; pg. 46, lines 23-25).

Claim 19 is dependent on claim 18 and specifies that the human c-Src tyrosine kinase inhibitor is selected from the group consisting of pyrazolopyrimidine PP1, pyrazolopyrimidine PP2, PD173955, PD162531, Radicol R2146 and Geldanamycin (pg. 5, lines 21-24; pg. 33, lines 14-16; pg. 46, lines 23-25).

Claim 20 is dependent on claim 18 and specifies that the human c-Src tyrosine kinase inhibitor is pyrazolopyrimidine PP1 (pg. 5, lines 22-23; pg. 33, lines 17-19).

Claim 32 is dependent on claim 18 and specifies that the human c-Src tyrosine kinase inhibitor is pyrazolopyrimidine PP2 (pg. 5, lines 22-23; pg. 33, lines 19-23).

6. Grounds of Rejection to be Reviewed on Appeal.

A. Claims 1, 2, 17 and 18 stand rejected under 35 U.S.C. 102(e) as being anticipated by US 6,001,839 ("the Calderwood Patent").

B. Claims 1, 2, 17 and 18 stand rejected under 35 U.S.C. 102(e) as being anticipated by US Patent Application No. 2003/0187001 ("the Calderwood Application").

C. Claims 1, 2, 17 and 18 stand rejected under 35 U.S.C. 102(e) as being anticipated by US Patent Application No. 2002/0156081 ("Hirst *et al.*").

D. Claims 3, 4, 19, 20, 32, and 33 stand rejected under 35 U.S.C. 103(a) as being obvious over the Calderwood Patent, the Calderwood Application, and Hirst *et al.*, in view of Hanke *et al.*

7. Argument.

I. Claims 1, 2, 17 and 18 Are Not Anticipated By the Calderwood Patent.

The rejection of claims 1, 2, 17 and 18 under 35 U.S.C. 102(e) as allegedly being anticipated by US 6,001,839 ("Calderwood Patent") is not warranted. "Anticipation requires that a single prior art reference describe each and every limitation of a claim either explicitly or inherently." *Atlas Powder Co. v. IRECO Inc.*, 51 USPQ2d 1943, 1945-46 (Fed. Cir. 1999). "Absence from the reference of any claimed element negates anticipation." *Rowe v. Dror*, 42 USPQ2d 1550, 1553 (Fed. Cir. 1997). The foregoing conditions for anticipation are not satisfied in this instance.

As pointed out in prior responses to Office Actions in this case, the Calderwood Patent teaches that certain pyrrolopyrimidine compounds are useful for treating VEGF-mediated edema. This patent only generally mentions the Src family of tyrosine kinases along with other classes of kinases (i.e., the Syk and Janus families, at col. 12, line 53, through col. 13, line 9). The present claims are limited to methods and articles of manufacture including inhibitors of *human c-Src*, a specific member of the Src family of tyrosine kinases. The portion of the Calderwood Patent that discusses determination of the *in vitro* potency of the pyrrolopyrimidine inhibitors (col. 18, line 28, through col. 19, line 18) teaches an assay for Lck (a Src family inhibitor) and Zap (a Syk family inhibitor), but does not teach or suggest activity against *human c-Src* for the disclosed compounds.

In addition, the Calderwood Patent is not enabling for a method of ameliorating

tissue damage related to vascular leakage or edema comprising contacting the tissue with a vascular permeability modulating amount of a pharmaceutical composition comprising a human c-Src tyrosine kinase inhibitor, as claimed in the present application. The Calderwood Patent merely contains a *general* teaching that the disclosed compounds are tyrosine kinase inhibitors, and specifically teaches that certain of the compounds are *Lck* inhibitors (col. 19, lines 12-14). As shown in McMahon *et al.*, *Current Opin. in Drug Discov & Devel.* 1(2):131-146 (1998), particularly at page 142 under heading "Summary and outlook;" (a copy of which is of record), the selectivity of tyrosine kinase inhibitors is highly unpredictable with large variability in selectivities and activities depending on the spacial arrangement of substituents.

The Calderwood Patent states that the pyrrolopyrimidines *may* be useful in treatment of "VEGF mediated edema," but provides no teaching whatsoever that an inhibitor of *human c-Src* would have such utility. For example, no activity data are presented in this reference for *any of the compounds disclosed*, against *any* tyrosine kinase, much less human c-Src. As noted above, there is no teaching or suggestion in the Calderwood Patent that the disclosed compounds are inhibitors of human c-Src or that c-Src inhibitors can be used to treat edema. Accordingly, this patent would not have enabled one of ordinary skill in the art to practice the methods and articles of manufacture claimed in the present application.

The Examiner refers to Burchat *et al.* as evidence that the compounds disclosed in the Calderwood Patent *inherently* are inhibitors of human c-Src. This reference has a date subsequent to the filing date of the present application, has not been applied against claims 1, 2, 17 and 18, and furthermore does not support the present rejection. Burchat *et al.* does not teach inhibition of *human c-Src*, in any event. The abstract of Burchat *et al.* (2000) merely states that "Compound 1 is orally active in animal models", but does not mention activity against human c-Src. On page 4 of the Office Action the Examiner alleges that the data in Table 2 of Burchat *et al.* (2000) shows that the compounds of the Calderwood Patent are Src kinase inhibitors. Table 2 includes a column labeled "src" with inhibition data thereunder; however, there is no indication in the table, or anywhere else in the reference, as to which "src" was allegedly used to obtain this data.

In fact, there are a number of different "src" kinases, as shown, for example in Thomas *et al.*, *Ann. Rev. Cell Dev. Biol.*, 1997; 13:513-609, which is of record as Exhibit C to the Declaration of David A. Cheresh, Ph.D., which was submitted along with the response to

the Office Action dated October 2, 2002. A copy of Thomas *et al.* is attached hereto, as Evidence Appendix A, for the convenience of the Board. Thomas *et al.* note that there are two distinct classes of "src", i.e., viral scr (v-Src) and cellular scr (c-Src) (see page 514, second paragraph); v-Src differs from c-Src in structure, as well as activity profile, however. For example, Y527 in c-Src is the primary site of tyrosine phosphorylation, *in vivo*, whereas the corresponding tyrosine is missing altogether in v-Src (see Thomas *et al.*, page 519, first full paragraph). Y527 is also important for the ability of c-Src to localize to focal adhesions (see page 530, second and third full paragraphs). Phosphorylation of Y527 prevents association of the enzyme at focal adhesions, whereas v-Src can associate with the focal adhesions without any activation (see page 530, third full paragraph.). v-Src also causes morphological alterations in focal adhesions in v-Src transformed cells, which is also observed in truncated Src (active), and activated mutants of c-Src, but not c-Src, *per se* (*Id.*).

In addition, the Burchat *et al.* reference does not specify the organism from which the "src" was obtained. It is known that Src kinases can differ significantly from species to species, as well. For example, the amino acid residue sequence of chicken c-Src differs significantly from that of human c-Src (see Fig. 2 and Fig. 4 of the present application, which show the amino acid residue sequences of chicken and human c-Src, respectively).

Accordingly, Burchat *et al.* (2000) does not cure the noted deficiencies of the Calderwood Patent as a reference against the present claims, since this reference does not show that the compounds disclosed in the Calderwood Patent are inhibitors of human c-Src.

For the reasons stated above, the Calderwood Patent does not anticipate claims 1, 2, 17 or 18. This rejection should be reversed.

II. Claims 1, 2, 17 and 18 Are Not Anticipated By the Calderwood Application.

The rejection of claims 1, 2, 17 and 18 under 35 U.S.C. 102(e) as allegedly being anticipated by US Patent Application No. 2003/0187001 ("Calderwood Application") is likewise not warranted. The Office Action indicates that the Calderwood Application, at paragraphs 56 and 101, discloses that certain pyrrolopyrimidine compounds are useful for treating VEGF mediated edema. The compounds of the Calderwood Application are described in a Markush structure, which begins at paragraph 36 and continues through paragraph 52. This Markush structure covers thousands (perhaps millions) of diverse

compounds. No data whatsoever is provided in this application to show that *any* of these compounds is active as an inhibitor of human c-Src, or of any other src-family kinase, for that matter. Nor does this reference teach that inhibitors of *human c-Src* can be used to treat edema. In fact, paragraph 56 merely makes a general statement regarding treatment of edema, while paragraph 101 more specifically indicates that compounds which inhibit *KDR* tyrosine kinase are useful for inhibiting vascular permeability and edema.

The Office Action (i.e., in last paragraph of page 4) states that the pyrrolopyrimidine compounds of the Calderwood Application are disclosed as inhibitors of "Scr kinases", citing paragraphs 53 and 111 for support. Paragraph 53 of the Calderwood Application only generally mentions the Src family of tyrosine kinases along with other classes of kinases (i.e., the Syk, Tec, Csk, Jak, Map, and Nik families); however, there are many members of the Src family of kinases. Human c-Src is not mentioned in this list. Similarly the laundry list of kinases in paragraph 111 mentions "Src", but does not indicate whether any of the compounds would be inhibitors of c-Src, much less human c-Src.

The present claims are limited to methods and articles of manufacture including inhibitors of *human c-Src*, which is not mentioned at all in the Calderwood Application. As noted above, the selectivity of tyrosine kinase inhibitors is highly unpredictable with large variability in selectivities and activities depending on the spacial arrangement of substituents (see, for example, McMahon *et al.*). The Burchat *et al.* reference does not remedy this deficiency in the Calderwood Application any more than it does the deficiencies of the Calderwood Patent. Thus, this reference would not have enabled one of ordinary skill in the art to practice the claimed invention with a reasonable expectation of success.

Accordingly, the Calderwood Application does not anticipate method claims 1 and 2 and article of manufacture claims 17 and 18, and this ground for rejection also should be reversed.

III. Claims 1, 2, 17 and 18 Are Not Anticipated By Hirst *et al.*

The rejection of claims 1, 2, 17 and 18 under 35 U.S.C. 102(e) as allegedly being anticipated by US Patent Application No. 2002/0156081 ("Hirst et al.") is likewise traversed. The Hirst *et al.* reference does not provide an enabling disclosure of the presently claimed invention. Treatment of edema is discussed by Hirst *et al.* only generally, in a laundry list shown in paragraph 315 of this lengthy application. In paragraph 350, Hirst *et al.*

state that some of the compounds disclosed therein can be used to treat edema. Of the over 900 examples of compounds presented in the 425 pages of Hirst *et al.*, there is not a single point of inhibition data presented to verify that any of the disclosed compounds are active against any tyrosine kinase. Only general allusions to unspecific activity against various diverse classes of tyrosine kinases are provided, as in paragraph 311. There is no specific teaching in Hirst *et al.* that an inhibitor of human c-Src can be used to treat vascular leakage and edema, as required by all of the present claims. The Examiner asserts, on page 7 of the Office Action, that Hirst *et al.* teach that each of the specific compounds disclosed in the reference can be used to treat edema. Clear support for this assertion is lacking, however. In addition, this reference does not teach or suggest that any of the compounds are inhibitors of human c-Src. Burchat *et al.* (2002) does not support the Examiner's contention that the compounds disclosed in Hirst *et al.* are inhibitors of human c-Src, for the same reasons as discussed above for the Calderwood references.

Thus, Hirst *et al.* cannot anticipate method claims 1 and 2 and article of manufacture claims 17 and 18. This rejection should be reversed, as well.

IV. Claims 3, 4, 19, 20, 32 and 33 Are Not Obvious Over the Calderwood Patent, the Calderwood Application and Hirst et al. in view of Hanke *et al.*

Claims 3, 4, 19, 20, 32 and 33 stand rejected under 35 U.S.C. §103(a) as being unpatentable over the Calderwood Patent, the Calderwood Application and Hirst *et al.* in view of Hanke *et al.* This rejection is unwarranted.

The test for obviousness is what the combined teachings of the references would have suggested to one of ordinary skill tin the art. *In re Young*, 927 F.2d 588, 18 USPQ2d 1089 (Fed. Cir. 1991). In order to establish a *prima facie* case for obviousness, all claim limitations must be taught or suggested by the prior art. *In re Royka*, 180 USPQ 580 (CCPA 1974). Additionally, "All words in a claim must be considered in judging the patentability of that claim against the prior art." *In re Wilson*, 165 USPQ 494, 496 (CCPA 1970). Furthermore, there must be a teaching in the references themselves that would have motivated one of skill in the art at the time the invention was made to combine the references with a reasonable expectation of success. *In re Vaeck*, 947 F.2d 488, 493, 20 USPQ2d 1438, 1442 (Fed. Cir. 1991). See also *In re Bell,* 26 USPQ2d 1529, 1531 (Fed. Cir. 1993); and *In re Fine*, 5 USPQ2d 1596, 1598 (Fed, Cir. 1988). That is not the case here. The combination of the

applied references would not have rendered the present claims obvious to one of ordinary skill in the art at the time the claimed invention was made.

Neither the Calderwood Patent, the Calderwood Application, nor Hirst *et al.* discloses the invention defined by claims 1, 2, 17 and 18, as noted above. Moreover, none of these references disclose the pyrazolopyrimidine inhibitors PP1 and PP2, which are called for by claims 3, 4, 19, 20, 32 and 33. Hanke *et al.*, while disclosing PP1 and PP2, does not disclose or suggest treatment of vascular leakage and edema utilizing an inhibitor of *human c-Src* as required by the present claims. Hanke *et al.* does demonstrate, however, that one inhibitor can have a wide variance in activity against different tyrosine kinases (see Table I on page 698). Hanke *et al.* would not have provided any motivation whatsoever to one of ordinary skill in the art to use PP1 or PP2 to ameliorate tissue damage due to edema or vascular permeability. At most, the teachings of Hanke *et al.* are but an invitation to experiment that does not vitiate patentability.

The contention that there is a structural similarity between the compounds disclosed in the Calderwood Patent and Calderwood Application to PP1 and PP2, and that this alleged structural similarity would have motivated one of skill in the art to use PP1 and PP2 to treat edema as allegedly described in the Calderwood references and Hirst *et al.*, is without merit. The alleged structural similarity between the Calderwood compounds and PP1/PP2 is superficial at best. These are clearly different chemical compounds. Pyrazolopyrimidines such as PP1 and PP2 are not pyrrolopyrimidines, and *vice versa*. As is evident from Hanke *et al.* and McMahon *et al.* discussed above, inhibition of tyrosine kinases is highly unpredictable. Small changes in structure can lead to large changes in activity and selectivity. The compounds of the Calderwood references are pyrrolopyrimidines, whereas PP1 and PP2 are pyrazolopyrimidines. The additional nitrogen in PP1 and PP2 relative to the Calderwood compounds could have a significant effect on activity and selectivity. In addition, the Calderwood compounds have a bulky phenoxy substituent on the phenyl ring, whereas PP1 and PP2 have relatively small methyl and chloro substituents on the phenyl ring. These differences could have significant effects on the binding affinity and selectivity of the inhibitors, particularly since the compounds bind to specific binding pockets in the enzymes (see McMahon *et al.*, page 135, paragraph bridging column 1 and column 2). Accordingly, one of ordinary skill in the art in March 2000 would not have had a reasonable expectation of success in using PP1 and PP2 of Hanke *et al.* to treat edema based on the Calderwood

references. Moreover, there is no suggestion in Hanke *et al.* that PP1 and/or PP2 are *human c-Src* inhibitors.

The alleged structural similarities between PP1 and PP2 to the compounds of Hirst *et al.* is also superficial. The hundreds of paragraphs of Markush description in Hirst *et al.* are almost incomprehensible with respect to what substituents are present on the pyrazolopyrimidine compounds disclosed therein. The structural formulas for the specific examples, shown on pages 82-102, 108-115, and at various other places within pages 125 through 388 of Hirst *et al.* provide some insight in this regard, however. All of the alleged inhibitor compounds specifically disclosed in this reference have a bulky aryl or heteroaryl ring attached to the phenyl substituent of the pyrazolopyrimidine. In contrast, PP1 and PP2 merely have a relatively small methyl or chloro substituent, respectively, on this phenyl ring. Similarly, PP1 and PP2 both have a t-butyl substituent on the nitrogen at the 7-position of the pyrazolopyrimidine ring, while the compounds disclosed in Hirst *et al.* have various cyclic substituents at this position, such as N-methypiperazinocyclohexyl, N-acyl piperadyl, N-alkyl piperadyl, substituted phenyl, piperadyl-substituted alkyl, and the like. Applicants are not aware of even a single example in Hirst *et al.*, which has a t-butyl substituent at the 7-position as found in PP1 and PP2.

The Examiner has relied upon *In re Ngai*, 70 U.S.P.Q. 1862 in rejecting Applicants arguments in support of the patentability of article of manufacture claims 19, 20, and 32. *In re Ngai* is readily distinguishable, however. The prior art in that case already taught a kit and the necessary components thereof (e.g., a 10x buffer therefor). That is not the situation here. The specific composition containing human c-Src tyrosine kinase inhibitor and capable of modulating vascular permeability increase as defined by these claims is not in the prior art, neither is a packaged version of that composition as claimed. The new printed matter unquestionably conveys new utility, a new feature, to the package, not previously known to one of ordinary skill in the art. This this case is inapposite to the present article of manufacture claims.

V. Conclusion.

All of the present claims are deemed patentable over the individual references and the combined teachings of the applied references, as well. Reversal of the rejections of claims 1-4, 17-20, 232, and 33 is requested. Allowance of all claims and early passage of the application to issue is solicited.

Respectfully submitted,

Dated 13 January 2001 — By _____
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CLAIMS APPENDIX

1. A method for ameliorating tissue damage related to vascular leakage or edema comprising contacting said tissue with a vascular permeability modulating amount of a pharmaceutical composition comprising a human c-Src tyrosine kinase inhibitor.

2. The method of claim 1 wherein said human c-Src tyrosine kinase inhibitor is a chemical inhibitor.

3. The method of claim 2 wherein said chemical inhibitor is selected from the group consisting of pyrazolopyrimidine PP1, pyrazolopyrimidine PP2, PD173955, PD162531, Radicol R2146 and Geldanamycin.

4. The method of claim 3 wherein said inhibitor is pyrazolopyrimidine PP1.

17. An article of manufacture comprising packaging material and a pharmaceutical composition contained within said packaging material, wherein said pharmaceutical composition is capable of modulating vascular permeability increase in a tissue suffering from a disease condition, wherein said packaging material comprises a label which indicates that said pharmaceutical composition can be used for treatment of vascular leakage or edema associated disease conditions, and wherein said pharmaceutical composition comprises a human c-Src tyrosine kinase inhibitor and a pharmaceutically acceptable carrier therefor.

18. An article of manufacture of claim 17 wherein said human c-Src tyrosine kinase inhibitor is a chemical inhibitor.

19. An article of manufacture of claim 18 wherein said human c-Src tyrosine kinase inhibitor is selected from the group consisting of pyrazolopyrimidine PP1, pyrazolopyrimidine PP2, PD173955, PD162531, Radicol R2146 and Geldanamycin.

20. An article of manufacture of claim 18 wherein said human c-Src tyrosine kinase inhibitor is pyrazolopyrimidine PP1.

32. An article of manufacture of claim 18 wherein said human c-Src tyrosine kinase inhibitor is pyrazolopyrimidine PP2.

33. The method of claim 3 wherein said human c-Src tyrosine kinase inhibitor is pyrazolopyrimidine PP2.

EVIDENCE APPENDIX A

THOMAS *ET AL.*

Annu. Rev. Cell Dev. Biol. 1997. 13:513–609

CELLULAR FUNCTIONS REGULATED BY SRC FAMILY KINASES

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KEY WORDS: Src, protein tyrosine kinases, tyrosine phosphorylation, receptors

ABSTRACT

Src family protein tyrosine kinases are activated following engagement of many different classes of cellular receptors and participate in signaling pathways that control a diverse spectrum of receptor-induced biological activities. While several of these kinases have evolved to play distinct roles in specific receptor pathways, there is considerable redundancy in the functions of these kinases, both with respect to the receptor pathways that activate these kinases and the downstream effectors that mediate their biological activities. This chapter reviews the evidence implicating Src family kinases in specific receptor pathways and describes the mechanisms leading to their activation, the targets that interact with these kinases, and the biological events that they regulate.

CONTENTS

1081-0706/97/1115-0513$08.00

EXHIBIT C

INTRODUCTION

Tyrosine phosphorylation has been implicated in the regulation of a variety of biological responses including cell proliferation, migration, differentiation, and survival. The protein tyrosine kinases involved in mediating these responses, as well as the receptors that activate them, encompass a diverse spectrum of proteins. Current evidence indicates that several distinct families of tyrosine kinases function in each of these responses and that additional complexity results from extensive cross-talk between different receptor pathways. One family of cytoplasmic tyrosine kinases capable of communicating with a large number of different receptors is the Src protein tyrosine kinase family (Src PTKs) (Figure 1).

The prototype member of the Src family protein tyrosine kinases was first identified as the transforming protein (v-Src) of the oncogenic retrovirus, Rous sarcoma virus (RSV) (Brugge & Erikson 1977, Purchio et al 1978). v-Src is a mutant variant of a cellular protein ubiquitously expressed and highly conserved through evolution (Stehelin et al 1976, Brown & Cooper 1996). A major breakthrough in understanding the function of the Src protein came from the finding that Src possesses protein tyrosine kinase activity (Collett & Erikson 1978, Levinson et al 1978). This evidence launched a search for related protein kinases, as well as investigations of the role of the viral and cellular forms of Src in the regulation of cell proliferation (for review, see Brown & Cooper

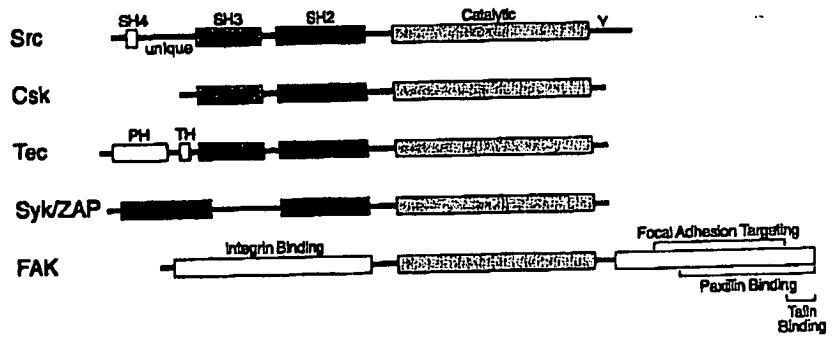

Figure 1 Domain structure of protein tyrosine kinases. Shown are the nonreceptor PTKs discussed in this chapter.

1996). Ten proteins were identified that contain structural features similar to Src and significant amino acid sequence homology: Fyn, Yes, Yrk, Blk, Fgr, Hck, Lck, Lyn, and the Frk subfamily proteins Frk/Rak and Iyk/Bsk (Cance et al 1994, Lec et al 1994, Thuveson et al 1995, Oberg-Welch & Welch 1995, Brown & Cooper 1996). Subsequent studies of these Src-related proteins led to the realization that these enzymes regulate many cellular events in addition to cell proliferation (e.g. cytoskeletal alterations, differentiation, survival, adhesion, and migration). This broad spectrum of activities is a consequence of the ability of these kinases to couple with many diverse classes of cellular receptors and many distinct cellular targets.

The focus of this review is on the structural and functional interactions between Src family kinases and cellular receptors and on receptor-induced biological activities regulated by these kinases. Before addressing these topics, we present a brief discussion of the expression of Src family kinases, their structural properties, and the role of individual domains in regulating the catalytic and binding activity of these kinases. For more detailed discussions of these topics, see a review by Brown & Cooper (1996).

Expression of Src Family Kinases

The Src PTKs can be subdivided into three groups based on their general pattern of expression (Table 1). Src, Fyn, and Yes are expressed in most tissues; however, individual kinases are expressed at elevated levels in certain cell types and some of these genes are expressed as alternatively spliced mRNAs in specific cell types. For example, Src is expressed ubiquitously; however, platelets, neurons, and osteoclasts express 5–200-fold higher levels of this protein (Brown & Cooper 1996).

Table 1 Expression of Src family kinases

Src	Ubiquitous; two neuron-specific isoforms
Fyn	Ubiquitous; T cell-specific isoform (FynT)
Yes	Ubiquitous
Yrk[a]	Ubiquitous
Lyn	Brain, B-cells, myeloid cells; two alternatively spliced forms
Hck	Myeloid cells (two different translational starts)
Fgr	Myeloid cells, B-cells
Blk	B-cells
Lck	T-cells, NK cells, brain
Frk subfamily	Primarily epithelial cells
Frk/Rak	
Iyk/Bsk	

[a]Only found in chickens.

The second group of Src PTKs, Blk, Fgr, Hck, Lck, and Lyn, are found primarily in hematopoietic cells (Bolen & Brugge 1997). Both Lck and Lyn have also been detected in neurons, suggesting that these kinases may function in additional cell types. As in the case of Src and Fyn, alternate isoforms of some of these proteins have also been identified.

Frk-related kinases represent a subgroup of Src-PTKs (Frk/Rak and Iyk/Bsk). Frk and Iyk kinases share homology in all regions, including the unique region, and are expressed predominantly in epithelial-derived cells (Cance et al 1994, Lee et al 1994, Oberg-Welsh & Welsh 1995, Thuveson et al 1995).

These observations indicate that all cells are likely to express multiple Src PTKs and potentially multiple isoforms of an individual member. In addition, within a cell, these kinases can be found in many different subcellular locations. For example, Src has been found in caveolae, focal adhesions, and endosomes, whereas other members such as Fgr and Frk have been found in the nucleus (Kaplan et al 1992, 1994, Cance et al 1994, Robbins et al 1995, Thuveson et al 1995, Li et al 1996b, Lowell & Soriano 1996). Thus Src PTKs can function in many distinct cells and in distinct subcellular locations.

Structural Domains of Src Kinases

Src PTKs are 52–62 kDa proteins composed of six distinct functional regions (Figure 1): (*a*) the Src homology (SH) 4 domain, (*b*) the unique region, (*c*) the SH3 domain, (*d*) the SH2 domain, (*e*) the catalytic domain, and (*f*) a short negative regulatory tail (Brown & Cooper 1996). The SH4 domain is a 15-amino acid sequence that contains signals for lipid modification of Src PTKs (Resh 1993). The glycine at position 2 is important for addition of a myristic acid moiety, which is involved in targeting Src PTKs to cellular membranes. This signal is absent in Frk (Lee et al 1994, Oberg-Welsh & Welsh 1995) but is present in Iyk. In addition, cysteine residues in the SH4 domain, which are present in all members except Src and Blk, are subject to palmitylation (Resh 1993). Frk and Iyk also have one of the conserved Cys residues, but whether these kinases are palmitylated has not been determined.

Following the SH4 domain is the unique domain which, as the name implies, is distinct for each member. The unique domain has been proposed to be important for mediating interactions with receptors or proteins that are specific for each family member. For example, sequences in the unique domain of Lck mediate its interaction with two T-cell surface molecules, CD4 and CD8 (Rudd et al 1988, Veillette et al 1988). Serine and threonine phosphorylation sites have also been identified in the unique domains of Src and Lck (Chackalaparampil & Shalloway 1988, Shenoy et al 1989, Morgan et al 1989, Winkler et al 1993). The precise function of these modifications is unclear but they may modulate protein:protein interactions or regulate catalytic activity.

The three domains that follow the unique region represent modular structures found in many classes of cellular proteins. The SH3 and SH2 domains are protein-binding domains present in lipid kinases, protein and lipid phosphatases, cytoskeletal proteins, adaptor molecules, transcription factors, and other proteins (Mayer & Baltimore 1993). The catalytic domain possesses tyrosine-specific protein kinase activity.

The SH3 domains of Src PTKs are composed of 50 amino acids (Pawson 1995, Cohen et al 1995). Alternatively spliced forms of Src, which contain 6- or 11-amino acid insertions in the SH3 domain, are expressed in CNS neurons (Brugge et al 1985, Martinez et al 1987, Sugrue et al 1990, Pyper & Bolen 1990). The SH3 domain is important for intra- as well as intermolecular interactions that regulate Src catalytic activity, Src localization, and recruitment of substrates.

SH3 domains bind short contiguous amino acid sequences rich in proline residues (Cohen et al 1995). All SH3 domain ligands contain a core consensus sequence of P-X-X-P; however, amino acids surrounding the prolines confer additional affinity and specificity for individual SH3 domains (Rickles et al 1995). SH3 ligands can bind in either a NH2 → COOH (Class I) or a COOH → NH2 (Class II) orientation (Yu et al 1994, Feng et al 1994). The SH3 binding pocket has two hydrophobic grooves that contact the core X-P-X-X-P sequence. A second region contacts the residues N-terminal (class I) or C-terminal (Class II) to the proline core. Binding affinities for SH3 domains and their ligands are in the micromolar range; however, such interactions may be strengthened in vivo by additional contacts between the target protein and other domains of Src (see below). Examples of proteins shown to interact with Src PTK SH3 domains either in vitro or in vivo include $p68^{sam}$, p85 phosphatidylinositol-3′

kinase (PI 3-K), and paxillin (Fukui & Hanafusa 1991, Liu et al 1993, Pleiman et al 1993, Prasad et al 1993a,b, Weng et al 1994, Taylor & Shalloway 1994).

A second modular domain that also controls the repertoire of proteins interacting with Src PTKs is the SH2 domain (Cohen et al 1995, Pawson 1995). Binding interactions mediated by the SH2 domain function in regulating the catalytic activity of Src PTKs, as well as the localization of Src or its binding proteins. In the case of Frk, a bipartite nuclear localization sequence is present in the SH2 domain and is likely to account for the ability of this kinase to localize to the nucleus (Cance et al 1994, Thuveson et al 1995). All SH2 domains bind to short contiguous amino acid sequences containing phosphotyrosine, and the specificity of individual SH2 domains lies in the 3–5 residues following the phosphotyrosine (+1, +2, +3, etc) (Songyang et al 1993, Pawson 1995). Amino acids preceding phosphotyrosine may also be important for regulating binding affinity (Bibbins et al 1993). Structural studies on Src family SH2 domains have shown that the ligand-binding surface of SH2 domains is composed of two pockets (Waksman et al 1993, Eck et al 1993). One pocket contacts the phosphotyrosine; the other pocket contacts the +3 amino acid residue following the phosphotyrosine. Src family kinases show a preference for leucine at this position (Songyang et al 1993). Examples of proteins shown to interact with the Src SH2 domain in vivo include the focal adhesion protein FAK (focal adhesion kinase), $p130^{cas}$, p85 PI 3-K, and $p68^{sam}$ (Fukui & Hanafusa 1991, Schaller et al 1994, Taylor & Shalloway 1994, Petch et al 1995).

Activation of Src Kinases

The SH2 and SH3 domains play a central role in regulating Src PTK catalytic activity. High-resolution crystal structures of human Src and Hck, in their repressed state, have provided a structural explanation for how intramolecular interactions of the SH3 and SH2 domains stabilize the inactive conformation of these kinases (see Figure 2) (Pawson 1997, Xu et al 1997, Sicheri et al 1997). The crystal structures include the SH3, SH2, and catalytic domains, and the negative regulatory tail. Both the SH3 and SH2 domains lie on the side of the kinase domain opposite the catalytic cleft. The SH3 and SH2 domains repress the kinase activity by interacting with amino acids within the catalytic domain, as well as with residues N-terminal and C-terminal, respectively, to the catalytic domain.

The SH3 domain interacts with sequences in the catalytic domain, as well as with sequences in the linker region that lies between the SH2 and catalytic domains (Sicheri et al 1997, Xu et al 1997). Although the linker region contains only a single proline residue, these sequences form a left-handed PPII helix and bind the SH3 domain in the same orientation as class II ligands. Two regions of the SH3 domain that flank the hydrophobic binding surface make contacts with the catalytic domain. Thus interactions with the linker region and the kinase

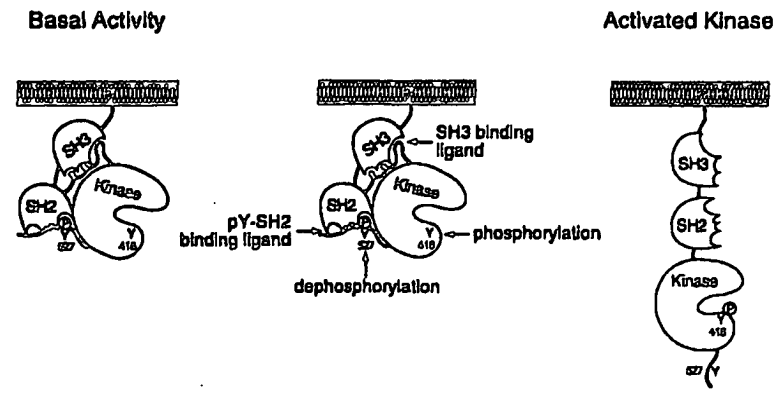

Figure 2 Mechanisms involved in activation of Src family kinases. The *left panel* shows a model of the structure of inactivated Src PTKs that are phosphorylated on the C-terminal tyrosine (Y527 in this model of Src). This model is based on the crystal structures of Src and Hck (Sicheri et al 1997, Xu et al 1997). The *middle panel* shows possible mechanisms involved in activation of Src PTKs. Y416 represents the autophosphorylation site in the activation loop of Src. The *right panel* represents a model for the activated state of Src in which the intramolecular interactions of the SH3 and SH2 domains are disrupted.

domain are likely to account for the SH3 domain's role in negatively regulating the catalytic activity of Src PTKs.

The SH2 domain interacts with pTyr 527 (Src) and adjacent residues in the negative regulatory tail (Brown & Cooper 1996). Y527 in c-Src, and the corresponding tyrosine in other Src PTKs, are the primary sites of tyrosine phosphorylation in vivo. This residue is phosphorylated by the cytoplasmic tyrosine kinase Csk (Cooper et al 1986, Okada & Nakagawa 1989, Nada et al 1991). Several lines of evidence indicate that loss of Y527 phosphorylation leads to activation of Src catalytic activity (Brown & Cooper 1996): (*a*) Mutation of Y527 results in constitutive activation of c-Src (Cartwright et al 1987, Kmiecik & Shalloway 1987, Piwnica-Worms et al 1987). (*b*) Y527 and several amino acids surrounding this residue are deleted in v-Src and similar truncations of c-Src cause activation of this enzyme (Reynolds et al 1987). (*c*) Disruption of the *csk* gene results in activation of at least three Src PTKs (Imamoto & Soriano 1993, Nada et al 1993). These results and others support a model whereby Csk-mediated tyrosine phosphorylation of the C-terminal tail promotes an intramolecular interaction between the SH2 domain and the phosphorylated tail, keeping the kinase in a closed, inactive conformation.

Some Src PTKs are not always phosphorylated at this negative regulatory tyrosine, yet remain relatively inactive. For example, in B cells, tyrosine

phosphorylation of the C-terminal tail of Lyn is barely detectable even though the catalytic activity of Lyn is not elevated. However, loss of Csk results in activation of Lyn (Nada et al 1993, Hata et al 1994). These results suggest that a balance between a tyrosine phosphatase and Csk is important for maintaining Lyn in an inactive state in unstimulated cells. In addition, other intramolecular interactions may be important for regulating the catalytic activity of Lyn. Although there are no real contacts between the SH2 and SH3 domain, the Src SH2 domain makes a few contacts with the large lobe of the catalytic domain, which may also contribute to repression of the kinase activity. Interactions between the SH3 domain and the kinase domain or upstream linker sequences may also contribute to repression of Lyn activity. For example, Hck which has been dephosphorylated, is further activated in the presence of an SH3 ligand (Moarefi et al 1997). Thus the role of C-terminal tail dephosphorylation in activation may vary in different systems.

Biochemical and structural studies of Src and other kinases suggest that the autophosphorylation site within the catalytic domain is also important for regulation of kinase activity. Analyses of the structures of the insulin receptor and protein kinase A (PKA) have shown that phosphorylation of analogous residues within the catalytic domain of these enzymes induces a conformational change that allows the kinase to assume an active conformation (Knighton et al 1991, Hubbard et al 1994, Johnson et al 1996). This site of phosphorylation corresponds to Y416 in c-Src, which is not phosphorylated in inactive wild type Src, but is constitutively phosphorylated in activated oncogenic Src mutants (Cooper et al 1986, Parsons & Weber 1989). Mutation of Y416 diminishes the transforming potential of both v-src and some oncogenic variants of c-Src, suggesting that phosphorylation of this residue may be important in vivo (Snyder et al 1983, Piwnica-Worms et al 1987, Kmiecik & Shalloway 1987, Kmiecik et al 1988). Structural studies of c-Src and Hck also suggest a possible regulatory role for phosphorylation of the catalytic domain (Pawson 1997, Xu et al 1997, Sicheri et al 1997). Protein kinase catalytic domains are composed of a small N-terminal lobe and a larger C-terminal lobe. The Src and Hck catalytic domains are in a closed conformation with the N- and C-lobes in close proximity, similar to that observed for active catalytic domains of PKA and the insulin receptor (Knighton et al 1991, Hubbard et al 1994, Johnson et al 1996, Pawson 1997). Although the Hck and Src catalytic domains appear to be in an active conformation, sequences in the N-terminal lobe are prevented from assuming a fully active conformation by constraints induced by the SH3/SH2/linker regions, the absence of tyrosine phosphorylation in the catalytic domain, and sequences in the C-terminal lobe. Thus phosphorylation of the activation loop tyrosine of Src PTKs is predicted to permit sequences in the N-terminal lobe to orient properly and allow the kinase to adopt an active conformation (under optimal conditions where restraints from SH3 and SH2 intramolecular interactions are disrupted).

Taken together, these studies suggest that there are multiple ways to activate Src family kinases (Figure 2). These include displacement of the intramolecular interactions of the SH2 or SH3 domains by high-affinity ligands or modification of certain residues, dephosphorylation of pY527 by a tyrosine phosphatase, or phosphorylation of Y416. As described below, more than one mechanism is often involved in Src activation in response to a single stimuli, and individual Src family members may be more sensitive to regulation by any one particular mechanism. This added complexity may influence temporal and spatial aspects of the regulation of Src PTKs and be important in determining which Src PTKs are activated by different stimuli. For example, in B cells, activation of a phosphatase that regulates the C-terminal tail of Lyn may not have a dramatic effect on Lyn activation (since only a small population of Lyn is phosphorylated at this site), but may be more important for activation of other Src PTKs expressed in these cells (e.g. Blk) (Hata et al 1994). In addition, regulation of the temporal aspects of Src kinase activation could affect the biological responses of the receptor. For example, transient or sustained activation of Src kinases could elicit distinct cellular responses following receptor activation. In PC12 cells, sustained MAP kinase activation correlates with a differentiation response, whereas transient MAP kinase activation correlates with a proliferative response (Marshall 1995).

In summary, the modular domains of Src PTKs endow these kinases with the ability to be regulated by and to communicate with a diverse group of proteins. The following sections provide an overview of the proteins that couple directly or indirectly with Src kinases. In particular, this review is focused on the different families of receptors that use Src PTKs to relay their messages and the downstream cellular events regulated by these kinases. Because of the vast amount of literature generated on the interactions between receptors and Src PTKs, we have concentrated on a subset of Src PTKs: Src, Fyn, and Lck. The review has been divided into three major sections that focus on (*a*) the interaction of Src PTKs with different receptor signaling pathways, (*b*) the cellular events in which Src PTKs are involved, and (*c*) the biological processes these kinases may regulate in vivo.

RECEPTOR PATHWAYS THAT COUPLE WITH SRC KINASES

Several strategies have been employed to determine if Src family kinases are involved in receptor-induced signal transduction pathways. These include investigations of whether receptor engagement leads to (*a*) coprecipitation or colocalization with the Src PTK, (*b*) activation of the Src PTK, or (*c*) phosphorylation of the Src PTK. In addition, investigations of whether inhibition of Src PTKs causes defects in receptor-induced changes in cell behavior have provided

evidence for involvement of these kinases in a receptor pathway. Because activation of one receptor pathway can lead to activation of other receptors, it has been difficult in certain systems to distinguish which receptor is responsible for Src kinase activation. For example, activation of G protein–coupled receptors (GPCRs), cadherins, integrins, and CAMs (immunoglobulin super family cell adhesion molecules), as well as stress pathways, result in activation or phosphorylation of receptor protein tyrosine kinases (RPTKs). Alternatively, activation of RPTKs or GPCRs can activate the binding activity of integrin receptors. In such systems, it is important to establish whether Src is activated following activation of the primary or secondary receptor pathway.

Although the receptors that couple with Src kinases are structurally and functionally distinct, a number of general conclusions can be drawn: (*a*) Src PTKs can interact directly or indirectly with receptors using a variety of different mechanisms. (*b*) Src PTKs can be activated by ligand engagement of the receptor, and activation can be mediated by multiple mechanisms. (*c*) Receptors not only regulate Src PTKs, but these kinases can also regulate the functional activity of receptors. The versatility of Src PTKs to function as both effectors and regulators of receptors allows these kinases to facilitate cross-talk between different receptors.

Immune Recognition Receptors and Major Histocompatibility Receptors

Immune recognition receptors (IRRs) play critical roles in immune responses to foreign substances and pathogens. This receptor family includes the T and B cell antigen receptors [TCR/CD3 and BCR) as well as Fc receptors (e.g. FcγRI, II, III, FcεRI, II) (Rudd et al 1993, Hulett & Hogarth 1994, Paolini et al 1994, Weiss & Littman 1994, Isakov et al 1994, Howe & Weiss 1995, Chan & Shaw 1995, DeFranco 1995, Bolen 1995, Roth & Wienands 1997, Daeron 1997). IRRs are composed of multiple subunits, some of which are involved in extracellular ligand interactions and others in coupling with intracellular cytoplasmic proteins. IRRs on T cells and B cells have served as prototypes for this family of receptors (Figure 3). Antigen recognition is mediated by surface IgM of the BCR or the α and β subunits of TCR/CD3. Intracellular signaling is mediated by the cytoplasmic domains of several receptor subunits (Igα and Igβ for the BCR and $\gamma, \delta, \epsilon, \zeta$ for TCR/CD3). Each of the latter subunits contains a shared sequence motif referred to as immunoreceptor tyrosine activation motif (or ITAM) defined by the sequence D- XX- Y-XX-L-X_{7-8}-Y-XX-L/I (Reth 1989). The tyrosine residues within this motif are phosphorylated following IRR engagement and play a critical role in recruitment of SH2 domain-containing kinases and other signaling proteins to the receptor complex.

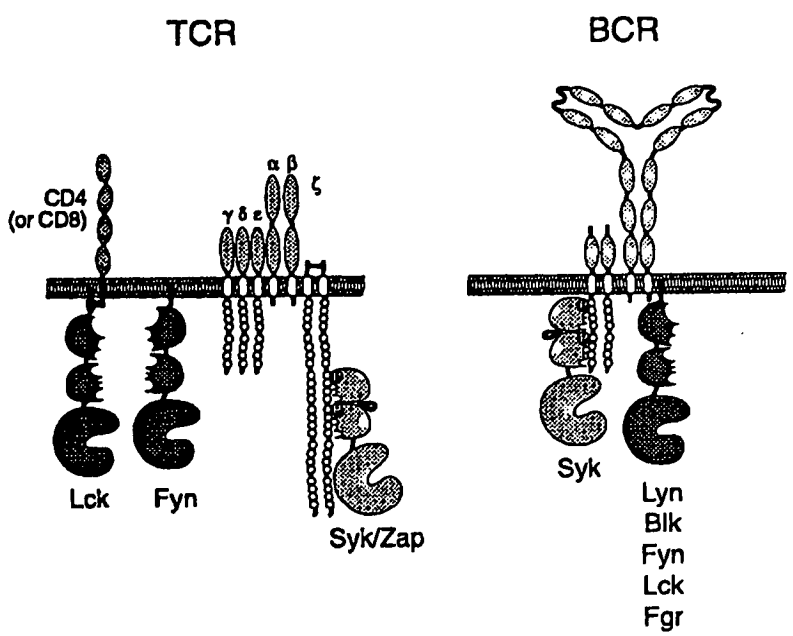

Figure 3 Components of the T cell receptor (TCR) and B cell receptor (BCR).

The specific kinases shown to couple with each member of the immune response receptor family are indicated in Table 2. There is a great deal of redundancy in Src kinase activation, both with respect to the ability of any one family member to be activated by multiple receptors and the ability of one receptor to activate multiple Src family kinases. This list of kinases activated by each receptor is likely incomplete because the full spectrum of kinases for each receptor has not been examined.

Antigen recognition by T cells involves coordination of the TCR/CD3 complex with one of two coreceptors, CD4 or CD8, which bind to major histocompatibility (MHC) class I or II proteins. Because antigens are presented to T cells in the form of peptides bound to the polymorphic cleft of MHC molecules, both the $\alpha\beta$ subunits of the TCR as well as CD4 or CD8 are required for recognition of the antigen-MHC complex (Townsend & Bodmer 1989).

In this section, we focus on Src kinases involved in antigen recognition by T cells because the role of protein tyrosine kinases is best characterized in this system and the TCR/CD3:CD4/8 coreceptor complex provides a model for how Src PTKs can coordinate signals transduced by two distinct receptors.

Table 2 Immune response receptor coupling with Src PTKs[a]

Receptor	Src PTK	Reference
TCR/CD3	Fyn, Lck	Rudd et al 1993, Isakov et al 1994, Weiss & Littman 1994, Bolen 1995, Howe & Weiss 1995
BCR	Lyn, Blk, Fyn, Fgr, Lck	Cambier & Jensen 1994, Desiderio 1994, Sefton & Taddie 1994, Penhallow et al 1995, Satterhwaite & Witte 1996
FcεRI	Lyn	Eisenman & Bolen 1990
FcεRII	Fyn	Sugie et al 1991, Maekawa et al 1992
FcγRIIA	Lyn	Bewarder et al 1996
FcγRIIb1	Fyn, Lyn	Sarmay et al 1995, Bewarder et al 1996
FcγRIIIA	Lck	Salcedo et al 1993
FcμR	Lck, Fyn, Lyn, Src	Rabinowich et al 1996

[a]Src family kinases are included in this table if there is any evidence suggesting that they can couple with an immune response receptor.

Src family kinases physically associate with both the TCR/CD3 and CD4/CD8 receptors. FynT coprecipitates with TCR/CD3 and its activity is stimulated following cross-linking of this receptor (Samelson et al 1990, 1992, Tsygankov et al 1992, Da Silva et al 1992). The FynT-TCR interaction is detected only if mild detergents are employed to maintain weak protein interactions. The precise nature of this interaction is not understood; however, the first 10 amino acids of FynT are necessary and sufficient to interact with the ζ TCR subunit, and transfer of this region from Fyn to Src conferred binding to ζ. FynT associates with other CD3 chains (ϵ, γ, ν) as well as ζ (Gauen et al 1992). Approximately 20% of FynT can be coprecipitated with TCR/CD3; however only 2–4% of total TCR/CD3 is associated with FynT (Sarosi et al 1992, Gassman et al 1992).

CD4 and CD8 directly couple with Lck, and cross-linking of these receptors leads to Lck activation (Rudd et al 1988, Veillette et al 1988, Barber et al 1989). This interaction, which is more stable in detergent extracts than the FynT-TCR interaction, is mediated by interactions involving C-X-C-P motifs from CD4 and CD8 and two Cys residues near the N terminus of Lck. The exact molecular nature of this interaction is unknown; however, it does not involve covalent bonding between the Cys residues (Shaw et al 1989, 1990, Turner et al 1990). Approximately 30–90% of Lck is stably associated with CD4 and CD8, depending on which population of T cells is examined and on the conditions employed for immunoprecipitation. An unrelated cytokine receptor, 4-1BB, which is induced following T-cell activation also possesses a C-X-C-P motif and has been shown to bind to Lck (Kwon et al 1987).

How are Src family kinases activated by IRRs? The mechanism of activation of Src kinases by immune response receptors is not completely understood

but involves a delicate balance between phosphorylation and dephosphorylation of these kinases. The protein tyrosine kinase Csk is important for negative regulation of Lck, Fyn, and other Src kinases that couple with IRRs. Loss of Csk leads to activation of Src family kinases (Imamoto & Soriano 1993, Nada et al 1993, Hata et al 1994) and prevents T and B cell maturation at an early stage of development (Gross et al 1995). Overexpression of Csk also suppresses TCR-induced protein tyrosine phosphorylation and IL-2 production in an antigen-specific mouse T cell line (Chow et al 1993) and prevents development of CD4+/CD8+ T cells when "knocked-in" to the *fyn* locus of *fyn-/-* mice (Takeuchi et al 1993, Kanazawa et al 1996). Expression of an activated Fyn mutant lacking the Csk phosphorylation site prevented Csk's inhibitory effects in a T cell line, suggesting that the Csk inhibition is mediated by suppression of Src kinases (Chow et al 1993).

The protein tyrosine phosphatase CD45 also plays a role in regulating Src family kinases through dephosphorylation. CD45 is able to dephosphorylate the C-terminal negative regulatory phosphorylation site (Mustelin et al 1990, 1992). CD45-deficiency in mice causes impaired development of CD4+/CD8+ T cells. T cells from these mice or cell lines lacking CD45 are defective in TCR stimulation of tyrosine phosphorylation, calcium mobilization, and IL-2 production, as well as anti-CD4 induction of tyrosine phosphorylation (Pingel & Thomas 1989, Koretzky et al 1990, 1991, Kishihara et al 1993). The defects in T cell signaling correlate with decreased catalytic activity of Lck and Fyn and increased phosphorylation of the C-terminal tyrosine (Ostergaard et al 1989, Volarevic et al 1990, Shiroo et al 1992, Mustelin et al 1992, Hurley et al 1993). CD45 deficiency can be overcome by co-ligation of CD4 and TCR, possibly from co-clustering of Lck and Fyn, which leads to kinase activation through transphosphorylation of the regulatory tyrosine in the catalytic cleft (Deans et al 1992). These results indicate that the status of Lck phosphorylation of the C-terminal tyrosine is balanced by the activity of Csk, CD45, and possibly other protein tyrosine phosphatases and that these regulatory enzymes play an important role in T cell receptor signaling.

Phosphorylation of the activation loop tyrosine is also important for activation of Src PTKs in T cells. Substitution of phenylalanine for this residue of Lck (Tyr 394) prevents activation by CD4 cross-linking (Veillette & Fournel 1990).

The pYXXL sequences within ITAMs resemble high affinity Src SH2-binding sites. Thus it is not surprising that Fyn and Lck have been reported to bind to these motifs in activated cells (Clark et al 1994, Flaswinkel & Reth 1994, Johnson et al 1995). It is possible that this interaction stimulates Src kinase activity by competitively interfering with the intramolecular negative regulatory interaction between the Src kinase SH2 domain and the phosphorylated C-terminal tyrosine residue. Because Src PTKs mediate phosphorylation of

ITAMs, this would not represent the primary mechanism for Src kinase activation through immune response receptors; however, it could contribute to further recruitment and activation of these kinases within receptor complexes.

Current models for the early events that are triggered following stimulation of the IRRs involve four different families of PTKs: Src kinases, Syk/ZAP, Tec kinases, and Pyk2. Src PTKs appear to be the primary kinases activated following engagement of these receptors and play a role in activation of the other PTK families (Isakov et al 1994, Weiss & Littman 1994, Bolen 1995, Chan & Shaw 1995, DeFranco 1995, Howe & Weiss 1995). Receptor clustering or dimerization leads to activation or recruitment of Src family kinases to the receptor complex. These kinases phosphorylate the tyrosine residues within the ITAM sequences of the receptor cytoplasmic domains. The phosphorylated ITAM sequences also serve as high-affinity binding sites for the tandem SH2 domains of Syk or ZAP, which redistribute to the receptor complex. Binding to ITAM sequences alone and/or phosphorylation by Src family kinases following binding leads to activation of the catalytic activity of Syk or ZAP. This activation leads to autophosphorylation of Syk/ZAP, which creates binding sites for interaction with SH2-containing proteins, and to phosphorylation of other cellular proteins, which transduce signals from the receptor. Activation of Tec kinases is also dependent on Src family kinases. Src kinase phosphorylation of Tec kinases leads to activation of autophosphorylation and exogenous substrate phosphorylation (Saouaf et al 1994, Rawlings et al 1996, Park et al 1996b). Lastly, activation of the FAK-related kinase Pyk2 (also called RAFTK) is also dependent on Fyn, as demonstrated by the absence of TCR-mediated Pyk2 phosphorylation or activation in Fyn-deficient mice (Qian et al 1997). Lck is not required for Pyk2 phosphorylation based on studies in Lck-deficient mice. Moreover, Fyn directly interacts with Pyk2 and activates its catalytic activity in T cells and in COS cells.

Because at least four PTK families are activated by IRRs, it is difficult to distinguish Src substrates from those of the other activated PTKs. However, there is significant experimental evidence indicating that Src kinases mediate phosphorylation of Syk/ZAP and Tec kinases, as well as IRR subunit ITAM sequences. The role of Src kinases in phosphorylation of ITAM residues has been shown by reconstituting the receptor complex in a transient expression system and through the use of somatic cell mutants of T cell lines. Phosphorylation of ITAM residues from a CD8-ζ fusion protein transfected into COS cells requires coexpression of a Src family kinase such as Fyn or Lck (Chan et al 1992). In contrast, ZAP expression in the absence of cotransfection of Src kinases was not sufficient to induce tyrosine phosphorylation of CD8-ζ. In addition, Jurkat T cell mutants lacking Lck are unable to phosphorylate ζ, and this activity is restored by transfection of Lck (Straus & Weiss 1992).

Two other proteins, LckBP1 and p68^{sam}, which are phosphorylated on tyrosine after TCR stimulation, also bind to Lck and can be coprecipitated with Lck from cell lysates (Takemoto et al 1995, Fusaki et al 1997). The identity of other Src substrates induced by IRRs is more ambiguous. PI 3-K has been shown to bind to Lck and FynT via both its SH3 and SH2 domains and may be a direct substrate of these kinases (Augustine et al 1991, Yamanashi et al 1992, Pleiman et al 1993). The adaptor protein Cbl and the inositol trisphosphate receptor (IP_3-R) are phosphorylated on tyrosine following TCR stimulation and coprecipitate with Fyn (Sawasdikosol et al 1996, Reedquist et al 1996, Tsygankov et al 1996, Tezuka et al 1996, Jayaraman et al 1996). Spleen cells from *fyn-/-* mice are deficient in TCR-induced Cbl phosphorylation, and Fyn overexpressing T cells show elevated Cbl phosphorylation, suggesting that Fyn is required for Cbl phosphorylation (Tezuka et al 1996). However, because Cbl also associates with ZAP and Syk, these PTKs may contribute to Cbl phosphorylation (Lupher et al 1996, Ota et al 1996, Panchamoorthy et al 1996). Other proteins phosphorylated on tyrosine in activated T cells include PLCγ, Vav, Shc, HS1, Lnk, ezrin, Slp 76, and p120^{rasGAP}, and its associated proteins p62 and p190. Other receptors such as CD5 and CD6 could also be substrates of any of the PTKs activated following T cell stimulation (Rudd et al 1993, Yamanashi et al 1993, Weiss & Littman 1994, Huang et al 1995).

Biochemical studies of T cells lacking Lck have allowed more precise dissection of the role of Lck in specific T cell signaling events (Straus & Weiss 1992, Karnitz et al 1992). TCR-induced phosphorylation of TCR-ζ, CD3-ϵ, and ZAP is defective in thymocytes from Lck-deficient mice (van Oers et al 1996a). In JCaM-1 Lck-deficient Jurkat T cells, which are defective in IL-2 production and calcium mobilization following TCR/CD3 stimulation, there is no detectable induction of tyrosine phosphorylation of ζ, ZAP, or other cellular proteins (Straus & Weiss 1992). Lck transfection can rescue the signaling defects in these cells. Although these cells express Fyn at levels close to the parental cells, this kinase does not appear to be sufficient for TCR signaling. Another line of Lck-deficient cells (derived from CTLL-2 cells) is profoundly deficient in cytolytic responses to TCR stimulation, but only modestly defective in induction of cell proliferation (Karnitz et al 1992). These results suggest that Lck is essential for activation of tyrosine phosphorylation through the TCR in certain populations of T cells. However, the specific mechanism for activation/recruitment of Lck to the TCR is not understood because Lck has not been shown to be associated with or activated by TCR clustering (Veillette et al 1989). It is possible that CD4/CD8-Lck may be recruited to the complex following TCR clustering; this has been reported in one T cell line (Burgess et al 1991). However, these JCaM1 cells express very low levels of CD4 and do not express CD8, so this Lck function may not involve CD4/CD8. It has been

suggested that Lck plays a role independent of CD4/CD8, most likely in phosphorylation of tyrosines in ITAM motifs, and that the availability of this distinct population of Lck for interaction with the TCR can affect the responsiveness of the receptor (Weiss & Littman 1994).

What is the physiological importance of Src kinases linking with both the TCR and CD4/CD8 proteins? Although cross-linking of the TCR/CD3 complex can lead to activation of IL-2 transcription, calcium mobilization, and DNA synthesis, co-clustering of CD4 and TCR/CD3 causes a dramatic increase in these responses (Eichmann et al 1987, Anderson et al 1987), and activation of T cell hybridoma cells by antigen-presentation leads to a 50–100-fold enhancement in responses relative to TCR cross-linking alone (Harding & Unanue 1990, Glaichenhaus et al 1991). The coordinate activation of Lck and Fyn through their interactions with CD4/CD8 and the TCR likely contributes to this synergistic activation of T cells. Under conditions of exposure to natural antigens in vivo, where only a small number of receptors are engaged (in some cases with low-affinity ligands), this may be critical for T cell biological responses.

There are several explanations for the enhanced responses induced by co-engagement of TCR/CD3 and CD4/CD8. One model derives from recent evidence that ZAP is constitutively associated with CD3-ζ in thymocytes (Watts et al 1994, Chan et al 1995, Wange et al 1995). Because ZAP activation (in contrast to Syk) is dependent on phosphorylation by Src family kinases, antigen-MHC stimulation of CD4/CD8-Lck may be required to trigger T cell responses by phosphorylating and activating ZAP. In addition, since only a small percentage of TCR/CD3 receptors are associated with Fyn (based on coprecipitation studies), CD4/CD8 co-clustering with TCR may play an important role in recruitment of a Src-family kinase to the TCR/CD3 complex to mediate ITAM phosphorylation and activate ZAP/Syk (Rudd et al 1993, Weiss & Littman 1994).

Other receptors expressed in T cells have also been shown to couple with Src family kinases and can contribute to the initial activation of T cells by antigen-presenting cells (e.g. CD2, CD28, Thy-1, Ly-6, LFA-1, CD43). CD2 has been reported to copurify with Lck and Fyn (Marie-Cardine 1992, Eljaafari et al 1994, Vitte-Mony et al 1994), and Lck is activated by CD2 ligation (Lacal et al 1990). Thy-1 and Ly-6 are glycosylphosphatidylinositol (GPI)-linked receptors that co-immunoprecipitate with Fyn and Lck (Brown 1993). CD43 cross-linking leads to association of Fyn with this receptor through the Fyn SH3 domains (Pedraza-Alva et al 1996). Other receptors, such as CD5, contain tyrosine residues that are phosphorylated after TCR stimulation and likely participate in antigen-stimulated activation events by recruiting signaling proteins like PI 3-K to the membrane via SH2-phosphotyrosine-mediated interactions (Raab et al 1994).

Thus Fyn and Lck are activated by antigen-MHC binding to the TCR and CD4/CD8. Both kinases participate in the earliest detectable receptor-mediated signal transduction events leading to activation of multiple downstream protein tyrosine kinases and other signaling proteins that mediate T cell activation events. Whether Src kinases play a direct role in any downstream cellular processes (e.g. secretion, cytoskeletal rearrangements, transcriptional activation, stimulation of DNA synthesis) or whether their role is primarily limited to phosphorylation of ITAMs and activation of other kinases that transduce signals responsible for these events remains to be elucidated. The role of Lck and Fyn in T cell development in vivo is discussed below.

Integrins and Other Adhesion Receptors

Adhesion to extracellular matrices and to other cells is mediated by a diverse family of receptors, the best-characterized being integrins, cadherins, selectins, and CAMs (Gumbiner 1993, Rosales et al 1995). Src kinases have been implicated in adhesion events regulated by these receptors by the evidence described below.

INTEGRINS Integrins are heterodimeric receptors that mediate cell-matrix and cell-cell interactions. There are at least 15 α subunits and eight β subunits that associate with each other to generate a diverse family of receptors with distinct ligand specificities (Hynes & Lander 1992). Following engagement by their adhesive ligands, integrins transduce signals within the cell that regulate cell adhesion and spreading, migration, proliferation, differentiation, and other changes in cell behavior. Src was first implicated in integrin-regulated events by the ability of oncogenic v-Src to phosphorylate β1 integrin, as well as several other proteins that are associated with integrin-nucleated focal adhesion complexes, e.g. paxillin, vinculin, talin, tensin, p130^{cas} and FAK (Sefton et al 1981, Hirst et al 1986, Pasquale et al 1986, DeClue & Martin 1987, Glenney & Zokas 1989, Reynolds et al 1989, Kanner et al 1990, Kanner et al 1991, Davis et al 1991, Sakai et al 1994). Subsequently, most of these proteins have been shown to be phosphorylated on tyrosine following natural engagement of different integrin receptors in non-transformed cells (Petch et al 1995, Nojima et al 1995, Clark & Brugge 1995, Schwartz et al 1995, Rosales et al 1995, Polte & Hanks 1995, Vuori & Ruoslahti 1995, Burridge & Chrzanowska-Wodnicka 1996, Lafrenie & Yamada 1996, Harte et al 1996). These early studies showing that integrin engagement induces tyrosine phosphorylation of a similar set of focal adhesion proteins as v-Src suggested that cellular Src PTKs were involved in integrin-induced tyrosine phosphorylation. Subsequent studies have shown that integrin engagement can activate Src kinase activity, that Src can localize to focal adhesion sites, and that Src associates with several proteins found in

focal adhesion complexes. This section discusses the evidence for Src kinase activation by integrins, possible mechanisms responsible for this activation, factors that affect Src localization to focal adhesions, and the nature of the interactions between Src and other focal adhesion proteins.

Src is activated transiently by engagement of integrins following cell attachment to a fibronectin matrix (Kaplan et al 1995). The mechanism responsible for Src activation has not been elucidated; however, this activation is preceded by dephosphorylation of the negative regulatory phosphorylation site pY527. Thus the initial activation of Src could be mediated by a tyrosine phosphatase that is activated by integrins or redistributed to cellular compartments containing Src. In addition, Src activation could be mediated, or at least stabilized, by interactions of the Src SH3 or SH2 domains with high-affinity binding sites on focal adhesion proteins. As discussed below, several focal adhesion proteins possess such Src SH2 and/or SH3 binding sites (including FAK, Cas, and paxillin), and several of these proteins coprecipitate with Src following integrin engagement.

The ability of Src to localize to focal adhesions is dependent on integrin-induced conformational changes that allow accessiblity of its SH2 and SH3 domains to other cellular proteins. When fibroblasts are plated on a fibronectin-coated surface, Src redistributes to newly formed focal adhesions following its activation (Kaplan et al 1995). Activation of Src and pY527 dephosphorylation is transient, peaking at 15 min; however, Src remains associated with the focal adhesions, suggesting that activation is not required for sustained localization at these sites. v-Src as well as activated mutants of c-Src or truncated, kinase-minus c-Src variants constitutively associate with focal adhesions (or podosomes, rosette-like adhesion sites found in transformed cells) when cells are plated in the absence of extracellular matrix (ECM) (Rohrschneider 1979, Shriver & Rohrschneider 1981, Krueger et al 1983, Kaplan et al 1994, Okamura & Resh 1994). These results suggest a model whereby integrin engagement leads to an unmasking of the Src SH3 and SH2 domains following pY527 dephosphorylation, which allows c-Src to associate with focal adhesion proteins (Figure 4). The SH2 and SH3 domains of mutant activated forms would not require ECM-induced unmasking since they would be constitutively open. Constitutive association of Src mutants with focal adhesions could lead to the morphological alterations in focal adhesions seen in v-Src-transformed cells and cells expressing the N-terminal truncation mutant of Src (1-251) (Kellie et al 1991, Kaplan et al 1994).

c-Src localization to focal adhesions requires myristylation and the SH3 domain, but not the SH2 or catalytic domains (Kaplan et al 1994). Although the SH3 domains and myristylation site are sufficient for Src focal-adhesion association (Kaplan et al 1994), the SH2 domain may participate in association with

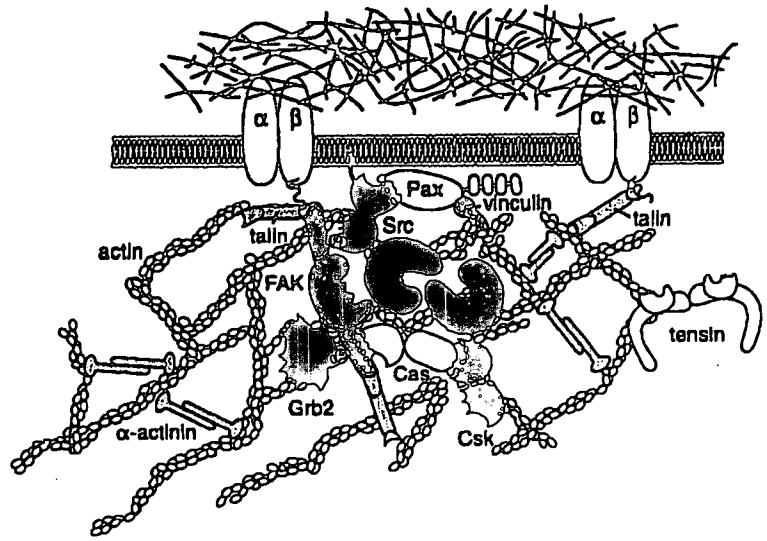

Figure 4 Src interaction with components of focal adhesion complexes. This figure was designed to indicate that Src associates with focal adhesions following engagement of integrins by extracellular matrix. The intermolecular interactions displayed are hypothetical, based on known interactions in vitro or co-immunoprecipitation from cell lysates and represent only a small number of proteins within these complexes.

integrin complexes through interactions with tyrosine phosphorylated proteins that localize to focal adhesions. One such protein is FAK (focal adhesion kinase), a protein tyrosine kinase that is phosphorylated and activated following engagement of many integrins (Schaller et al 1992, Guan & Shalloway 1992, Kornberg et al 1992, Lipfert et al 1992, Schaller & Parsons 1993). FAK coprecipitates with Src and Fyn and this coprecipitation is dependent on an intact FAK autophosphorylation site, Y397 (Schaller et al 1994, Cobb et al 1994, Xing et al 1994, Eide et al 1995). Y397 is part of a pY-A-E-I motif that binds to Src/Fyn/ Yes SH2 domains with a higher affinity than the pY527 site at the C terminus of Src. Thus it is possible that the Y397 FAK autophosphorylation site competes with the Src C-terminal phosphotyrosine site, thereby leading to activation of Src (through disruption of the intramolecular interaction).

It has been postulated that Src association with FAK may facilitate Src-mediated phosphorylation of other tyrosine residues on FAK, some of which serve as binding sites for additional SH2-containing proteins (Schaller et al 1994) (Figure 5). For example, phosphorylation of Y397 is required for phosphorylation of FAK on Y925, a Grb2-binding site and phosphorylation of Y407,

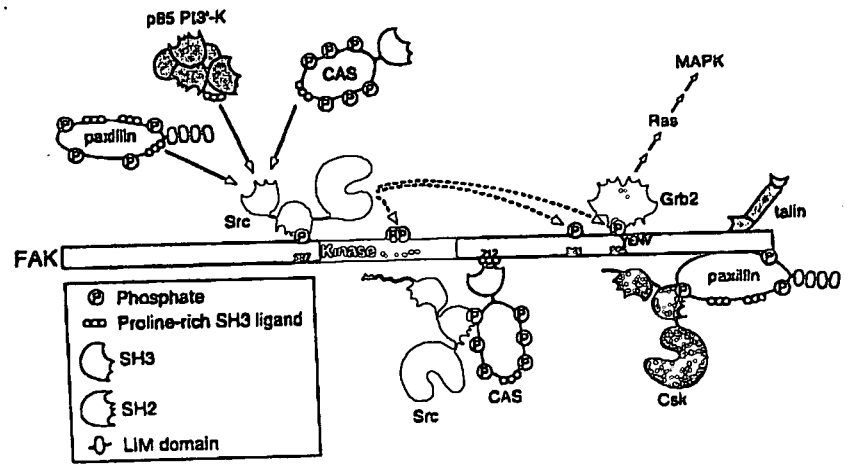

Figure 5 Interactions between FAK, Src, and other focal adhesion proteins.

Y861, Y576, and Y577, the latter two sites being required for maximal kinase activity of FAK and for FAK:p130^{cas} complex formation (Schlaepfer et al 1994, Calalb et al 1995, 1996, Schlaepfer & Hunter 1996, Polte & Hanks 1997). Thus Src or Fyn interaction with FAK may play an important role in FAK's interaction with other cellular proteins and in regulating FAK activity.

Several of the proteins that associate with FAK may be substrates of Src. FAK appears to serve as a scaffold to organize a network of signaling and cytoskeletal proteins. As shown in Figure 5, FAK associates with p130^{cas}, paxillin, Src, Grb2, and has also been shown to bind to p85:PI 3-K. p130^{cas}, paxillin and PI 3-K could also interact with Src through SH3 domain interactions (Hildebrand et al 1993, Polte & Hanks 1995, Chen et al 1995, Harte et al 1996, Burnham et al 1996, Vuori et al 1996, Schaller et al 1994). Tyrosine phosphorylation of paxillin following adhesion of cells to fibronectin requires Y397, suggesting a role for Src family kinases; however, because paxillin is phosphorylated in *src-/-* fibroblasts, it appears that Fyn or another PTK can compensate for the absence of Src (Schaller & Parsons 1995, Hamasaki et al 1996). Supporting this possiblity is the evidence that paxillin phosphorylation is significantly reduced in *src-/-; fyn-/-* double mutant cells (S Thomas, unpublished results). p130^{cas} is not phosphorylated in *src-/-* fibroblasts, suggesting that Src is involved in phosphorylation of this protein. However, the non-catalytic domain of Src may indirectly regulate p130^{cas} phosphorylation by mediating its localization to focal adhesions (Bockholt & Burridge 1995, Hamasaki et al 1996, Vuori et al 1996, Schlaepfer et al 1997; K Vuori, unpublished results). p130^{cas} phosphorylation can be rescued by expression of a kinase-minus truncated form of Src (Schlaepfer et al 1997).

FAK may directly or indirectly recruit Csk to focal adhesions. Csk, which phosphorylates the negative regulatory tyrosine in the C-terminal tail of Src family kinases, also localizes to focal adhesions (Howell & Cooper 1994, Bergman et al 1995). Csk relocates to adhesion plaques when Src is activated, and redistribution to these sites requires the SH2 and SH3 domains of Csk (Howell & Cooper 1994). FAK and paxillin contain binding sites for the Csk SH2 and possibly SH3 domains (Sabe et al 1994). These data led to the proposal of a model suggesting that Csk is recruited to the focal adhesion by tyrosine phosphorylated focal adhesion proteins, possibly FAK or paxillin. Following this redistribution, Csk could phosphorylate Src on Y527, possibly leading to inhibition of Src catalytic activity (Sabe et al 1994, Howell & Cooper 1994).

A close relative of FAK, referred to as Pyk2, RAFTK, or CAKβ, undergoes an adhesion-dependent increase in tyrosine phosphorylation when cells are plated on fibronectin-coated dishes in some cell types, colocalizes with vinculin in an adherent human megakaryocytic cell line, and coprecipitates with paxillin in B cells (Lev et al 1995, Sasaki et al 1995, Li et al 1996a, Salgia et al 1996). The Y-A-E-I site is conserved between Pyk2 and FAK and has been shown to couple with Src in bradykinin and LPA signaling pathways in PC12 cells (Dikic et al 1996). Pyk2 is also regulated by Ca^{2+}, protein kinase C, and stress pathways (Lev et al 1995, Siciliano et al 1996, Tokiwa et al 1996, Yu et al 1996). Pyk2 expression is more restricted than FAK; however, in those cells in which both kinases are expressed, there may be redundant as well as unique functions of these kinases.

Other integrin receptors Although integrin receptors for fibronectin are better characterized than others with respect to their coupling with Src PTKs, these kinases have been linked to other integrins as well. In platelets, Src associates with integrin-dependent detergent-insoluble cytoskeletal complexes following platelet aggregation, which is mediated by the integrin $\alpha_{IIb}\beta_3$ (Horvath et al 1992, Clark & Brugge 1993, Pumiglia & Feinstein 1993). This association with detergent-insoluble material is not detected in platelets from patients having mutations in the platelet integrin receptor $\alpha_{IIb}\beta_3$. Thrombin treatment of platelets causes an early integrin-independent activation of Src and pY527 dephosphoryation; however, Src does not associate with integrin complexes until after platelet aggregation mediated by $\alpha_{IIb}\beta_3$. Thus Src activation is uncoupled from integrin activation in this system.

Src is also activated by binding of osteoclasts and melanoma cells to osteopontin mediated by $\alpha_V\beta_3$ integrins. Src coprecipitation with this integrin following osteopontin binding requires the cytoplasmic tail of the α_V chain.

However, it is not known whether this interaction is direct or through other α_V-binding proteins (Hruska et al 1995, Chellaiah et al 1996). Src-deficient osteoclasts show defects in calcium signaling and in the induction of tyrosine phosphorylation following engagement of $\alpha_V\beta_3$, suggesting that Src-dependent events in integrin adhesion pathways may contribute to the resorption defect in Src-/- mice (R Baron, J Levy, unpublished results).

The role of Src family members other than Src and Fyn in integrin signaling has not been explored extensively. In neutrophils, engagement of $\beta 2$ integrins leads to activation of Fgr, which is enhanced by treatment with tumor necrosis factor (TNF) in a $\beta 2$-dependent fashion. Antibodies to $\beta 2$ block TNF activation of Fgr, and activation of Fgr in response to TNF does not occur in neutrophils from patients lacking $\beta 2$ (Berton et al 1994). In addition, analysis of neutrophils from *hck-/-; fgr-/-* mice showed that Hck and Fgr are required for signaling through leukocyte $\beta 2$ and β_3 integrins leading to neutrophil spreading and respiratory burst (Lowell et al 1996a). These results suggest that Fgr couples with $\beta 2$ integrins following their activation by TNF and binding to adhesive ligands.

Integrin coreceptors Accumulating evidence suggests that other cellular membrane proteins couple with integrins to modulate their adhesive functions or merely to exploit the ability of integrins to communicate with the intracellular environment. Several GPI-linked receptors (uPAR, CD14 and FcγRIIIB/CD16), as well as TM4 proteins, GPIV, IAP, and caveolin, either couple directly with integrins or show functional interactions with them (Brown et al 1990, Huang et al 1991, Asch et al 1991, Fukasawa et al 1995, Petty & Todd 1996, Wary et al 1996, Li et al 1996b, Dorahy et al 1996, Gao et al 1996, Berditchevski et al 1996). Although the precise roles of these associated membrane proteins have not been defined, the co-modulatory activity of some may involve Src family protein tyrosine kinases. Several types of interactions have been reported: (*a*) physical interaction between the receptor and Src PTK [e.g. GPIV, which forms tight complexes with Fyn, Lyn and Yes in platelets (Huang et al 1991); caveolin, which interacts directly with c-Src and coprecipitates with α integrin subunits (Li et al 1996b, Wary et al 1996)]; and (*b*) indirect activation of Src kinases through integrins [e.g. IAP, which strongly enhances integrin-mediated tyrosine phosphorylation (Gao et al 1996)]. Integrin engagement has also been shown to lead to phosphorylation of receptor protein tyrosine kinases such as the platelet-derived growth factor (PDGF) receptors. Because Src kinases phosphorylate receptor protein tyrosine kinases (RPTKs) and act downstream from these receptors, Src could be involved in integrin-mediated coupling with these receptors (Wasilenko et al 1991, Hansen et al 1996, Peterson et al 1996).

CAMs Neurite extension on L-CAM and N-CAM is reduced 50% in neurons from *src-/-* or *fyn-/-* mice, respectively (Beggs et al 1994, Ignelzi et al 1994), suggesting that these kinases may be involved in signaling through these CAM family receptors. Fyn coprecipitates with the NCAM140 isoform, which is predominantly localized in migrating growth cones, but not with NCAM180 or NCAM 120 (Beggs et al 1997). Src does not coprecipitate with any NCAM isoform. These results suggest that NCAM isoforms may couple with specific Src family kinases. In contrast, L1-CAM stimulation transiently activates both Src and Fyn in P19 embryonic carcinoma cells (Takayama et al 1997). Inhibition of these kinases through overexpression of Csk results in a defect in neurite fasciculation and cell-to-cell aggregation, implying that Src kinases may play roles in cell interactions mediated by CAM receptors. Src family kinases may be indirectly involved in CAM cell adhesion through their activation by FGF receptors. CAM-induced neurite outgrowth is dependent on activation of fibroblast growth factor (FGF) receptors (Williams et al 1994, Saffell et al 1997). As discussed below, Src associates with FGFR and is activated following treatment with FGF (Zhan et al 1994, Landgren et al 1995). In addition, microinjection of an antibody to Src inhibits FGF-induced neurite outgrowth in PC12 cells (Kremer et al 1991).

CADHERINS Engagement of cadherin receptors has not been reported to activate Src-related kinases; however, Src has been shown to localize to cell-cell contacts with cadherin in aggregated cytotrophoblasts, and Src is activated significantly when cytotrophoblasts are maximally activated and starting to fuse (Rebut-Bonneton et al 1993). In addition, cadherins and their associated catenin proteins (α, β, plakoglobin) are phosphorylated on tyrosine in Src-transformed cells (Reynolds et al 1992, Matsuyoshi et al 1992, Hamaguchi et al 1993). v-Src expression causes a weakening of cadherin-mediated cell adhesion; however, it is difficult to definitively link this to catenin phosphorylation because many other cytoskeletal proteins are also phosphorylated in v-Src-expressing cells (Matsuyoshi et al 1992, Takeda et al 1995). These studies raise the question whether Src is involved in normal modulation of the cadherin adhesiveness or in downstream events that are triggered by cadherin receptor signaling. As with N-CAM and L-CAM, there is evidence suggesting that neurite outgrowth of rat cerebellar neurons induced by N-cadherin are also mediated by FGF receptors (Williams et al 1994). In other cell types, epidermal growth factor receptor (EGFR) colocalizes with cadherins at cell-cell adherens junctions, and in vitro association assays suggest that β-catenin mediates the interaction with EGFR through its highly conserved central core (Rebut-Bonneton et al 1993, Hoschuetzky et al 1994). Thus cadherins may recruit Src family kinases indirectly through activation of RPTKs.

SELECTINS Selectin interactions with their glycoprotein ligands mediate initial steps in leukocyte adhesion to endothelial cells (Springer 1995). Selectin engagement of neutrophils and T cells induces tyrosine phosphorylation of several cellular proteins and activates MAP kinase and O_2^- synthesis (Waddell et al 1995, Brenner et al 1996). In T cells, selectin engagement results in activation of Lck, and induction of the cellular responses described above requires Lck since they were defective in Lck-deficient JCaM1.6 cells and rescued by transfection of Lck (Brenner et al 1996).

Src kinases are activated following engagement of multiple receptor pathways that regulate cell:cell and cell:matrix interactions. The roles of Src kinases in cellular events that are regulated by adhesion receptors are discussed below.

Receptor Protein Tyrosine Kinases

The receptor protein tyrosine kinase (RPTK) family is a diverse group of transmembrane proteins that bind to soluble and transmembrane ligands. Ligand binding results in stimulation of the catalytic activity of the receptor and initiates a cascade of signaling events that coordinate the diverse spectrum of biological responses mediated by these receptors (Kazlauskas 1994, van der Geer & Hunter 1994). RPTKs recruit multiple signaling proteins, including additional tyrosine kinases, to serve as downstream effectors. Among the kinases that participate in RPTK signaling are Src PTKs (Erpel & Courtneidge 1995).

Src family PTKs appear to communicate with many different RPTKs (Table 3). The biochemical connections between these different receptors and Src family kinases include phosphorylation of Src family PTKS, association with the RPTK, activation of Src PTKs, and phosphorylation of the RPTK.

Table 3 RPTKs coupled to Src PTKs

Receptor	Src PTK	Reference
PDGF-Rα/β	Src, Fyn, Yes	Kypta et al 1990, Twamley et al 1992; A Kazlauskas, personal communication;
EGF-R	Src, Fyn, Yes	Luttrell et al 1988, Sato et al 1995, Weernink & Rijksen 1995, Roche et al 1995b
FGF-R	Src	Zhan et al 1994
CSF-1R	Src, Fyn, Yes	Courtneidge et al 1993
NGF-R	Src	Kremer et al 1991
HGF-R	Src	Faletto et al 1993, Grano et al 1996
IR	Fyn	Sun et al 1996
IGF-R	Src	Kozma & Weber 1990, Peterson et al 1996
Neu (ErbB2)	Src	Muthuswamy & Muller 1995

PDGF RECEPTOR The first RPTK to be linked with Src PTKs was PDGF-Rβ (Ralston & Bishop 1985). Treatment of fibroblasts with PDGF BB causes an increase in Src, Fyn and Yes catalytic activity. In addition, these kinases can bind directly to PDGF-Rβ and receptor activation induces both serine and tyrosine phosphorylation of Src and Fyn (Kypta et al 1990, Gould & Hunter 1988). Src PTKs are also activated in response to PDGF AA and can associate with the α-receptor (A Kazlauskas, personal communication).

Association Src PTKs can bind directly to the PDGF-R (Kypta et al 1990, Twamley et al 1992). The Src SH2 domain binds to two phosphotyrosine residues in the juxtamembrane region of the PDGF-R (pY579 and pY581 for β and pY572 and pY574 for α) (Mori et al 1993; A Kazlauskas, personal communication). Mutation of both residues ablates the interaction between Src PTKs and the receptor. In the case of the β-receptor, interpretation of this result is complicated by the fact that this mutant receptor is catalytically compromised and, therefore, phosphorylation of other sites on the receptor is affected (Mori et al 1993). However, a similar mutation in the α-receptor ablates the interaction with Src PTKs without affecting its catalytic activity (A Kauzlaskas, personal communication). In addition, Src can directly bind a phosphopeptide comprising residues 572–589 of the β-receptor (Mori et al 1993, Alonso et al 1995). These results are consistent with a role for the juxtamembrane tyrosines of the receptor in mediating the interaction with Src PTKs.

Activation Association of Src PTKs with the PDGF-R is likely to serve as an initial mechanism for activation of these kinases. Consistent with this hypothesis, Src can be activated in vitro with the tyrosine phosphorylated juxtamembrane peptide (β-receptor), and mutation of the juxtamembrane tyrosines in the α-receptor prevents PDGF-induced activation of Src (Mori et al 1993, Alonso et al 1995; A Kauzlaskas, personal communication). Thus PDGF-stimulated autophosphorylation would create a binding site for the Src SH2 domain, which would displace the intramolecular interaction with the negative regulatory tail and activate Src.

PDGF-induced phosphorylation of Src may also play a role in activation of Src PTKs. Src is phosphorylated on two serine residues, a tyrosine residue(s) in the N-terminal half and a tyrosine residue in the catalytic domain (Y416) in response to PDGF treatment (Ralston & Bishop 1985, Gould & Hunter 1988, Stover et al 1996, Broome & Hunter 1997).

One of the PDGF- and insulin-induced sites of serine phosphorylation is Ser12, which can be phosphorylated by PKC in vitro and is also phosphorylated in vivo in response to TPA treatment (Gould & Hunter 1988, Gould et al 1985). Phosphorylation of this residue does not appear to have any effect on the in vitro kinase activity of Src, and studies in other systems have shown that mutation

of Ser12 does not affect the transforming potential of an activated Src variant (Gould et al 1985, Parsons & Weber 1989). These studies suggest that Ser12 plays only a minor, if any, role in PDGF-mediated activation of Src. Because the other site of serine phosphorylation has not been mapped, the role of serine phosphorylation in PDGF-R-mediated Src activation remains elusive.

Two different tyrosine residues in the N-terminal half of Src have been identified as PDGF-induced phosphorylation sites. This discrepancy could be due to differences in the experimental approaches used to determine the site of phosphorylation. In one study Tyr213 was defined as the major site of tyrosine phosphorylation in PDGF-treated cells (Stover et al 1996). A tryptic peptide containing Tyr213 of Src can be phosphorylated by the PDGF-R in vitro, and this peptide co-migrates with a tryptic peptide obtained from Src isolated from PDGF-treated cells. Tyr213 is located within the SH2 domain of Src near the phosphopeptide-binding site. Src phosphorylated at this site in vitro by the PDGF-R has a reduced ability to bind a peptide corresponding to the negative regulatory C-terminal tyrosine (pYQPGE) but is unaffected in its ability to bind a phosphopeptide corresponding to a previously identified EGF-R binding site (pYDGIP). It has been postulated that the presence of the proline in the +2 position of the negative regulatory C-terminal peptide would promote an interaction between the glutamic acid in position +4 with Arg205 of the SH2 domain. Arg205 is in close proximity to Tyr213. Thus phosphorylation of Tyr213 could disrupt the SH2:pYQPGE interaction involving Arg205 and the glutamic acid in the COOH terminus of Src, resulting in a conformational transition from the closed to open state. Such a model argues that phosphorylation of Tyr213 would play a role in PDGF-mediated activation of Src. Interestingly, the corresponding residue in Lck (Tyr192) is tyrosine phosphorylated after T-cell activation and is proposed to play a role in Lck activation (Couture et al 1994). Mutational studies to determine whether loss of this site affects PDGF-mediated tyrosine phosphorylation or activation of Src will be important for testing this model and for definitively showing whether this residue is phosphorylated in vivo.

In contrast to the above results, in another study, Tyr138 was shown as the major PDGF-induced tyrosine phosphorylation site on Src (Broome & Hunter 1996, 1997). This site is phosphorylated in vitro by the PDGF receptor and is also phosphorylated in vivo after PDGF treatment of fibroblasts. In addition, mutation of Tyr138 abolishes (*a*) PDGF-mediated tyrosine phosphorylation of Src in vivo, (*b*) coprecipitaton of Src with the PDGF-R in NP40 cell extracts, and (*c*) PDGF-induced DNA synthesis. This mutant, however, can still be activated by the PDGF-R. Thus phosphorylation of Tyr138 is unlikely to play a role in the activation of Src by the PDGF-R; instead binding studies suggest that this site plays a role in regulating ligand binding to the SH3 domain. Tyr138 is located in the peptide binding groove of the SH3 domain and likely contacts SH3 ligands. Phosphorylation of this residue results in a significant decrease in the ability of the SH3 domain to bind to either class I or class II peptide ligands (Broome & Hunter 1996). These results suggest that phosphorylation of Tyr138 is unlikely to play a direct role in PDGF-mediated activation of Src, but could regulate substrate specificity or, alternatively, provide a binding site for an SH2 ligand.

Although the site(s) of tyrosine phosphorylation in the N-terminal half of Src and its role in activation has not been completely resolved, tyrosine phosphorylation in the catalytic domain is likely to contribute to the PDGF-mediated increase in Src catalytic activity. Phosphorylation of the autophosphorylation site in Src (Y416) can be detected after PDGF treatment using an antiserum that specifically recognizes this phosphorylation site (X Zhou, S Sarkar & J Brugge, unpublished results). Since mutational studies suggest that phosphorylation of Y416 plays a role in regulation of its catalytic activity, phosphorylation of this tyrosine could be important for PDGF-induced activation of Src PTKs. Expression of the Y416F mutant in Src-deficient cells and analysis of PDGF-induced Src activation may help to address this issue.

EGF-RECEPTOR Src is also involved in EGF-R signaling. Overexpression of Src enhances many different EGF responses including DNA synthesis, protein tyrosine phosphorylation, and tumor formation in nude mice (Luttrell et al 1988, Wilson et al 1989, Wilson & Parsons 1990, Chang et al 1995, Maa et al 1995). EGF treatment induces a two- to threefold increase in Src catalytic activity and translocation of Src to a Triton-insoluble fraction, another hallmark of Src activation (Sato et al 1995a, Weernink & Rijksen 1995).

Association/activation Although Src has been shown to associate with the EGF-R in some cell systems, the nature of this interaction and its role in the initial activation is unclear. Sequences in the catalytic domain, as well as the SH2 domain of Src, have been proposed to mediate interactions with the EGF-R. A peptide corresponding to residues 413–431 from the catalytic domain can partially dissociate a constitutive interaction between the EGF-R and Src. There is no evidence, however, that this region of Src interacts directly with the EGF-R (Sato et al 1995a). In addition, peptides encompassing several receptor autophosphorylation sites bind to the Src SH2 domain in vitro. Tyr891 has also been shown to interact with Src both in vitro and in vivo (Sierke et al 1993, Stover et al 1995). Src can also phosphorylate Tyr891 and several other sites in vitro (Sato et al 1995, Lombardo et al 1995). These results, together with previous observations that the EGF-R is phosphorylated on novel tyrosine residues when Src is overexpressed with the EGF-R (Maa et al 1995), suggest that Src can potentially phosphorylate and bind to the EGF-R.

As with PDGF-induced Src activation, the initial activation of Src by EGF has been postulated to be mediated by Src's interaction with the receptor. Binding of a phosphorylated peptide encompassing pY891 to Src increases its catalytic activity; however, since Y891 has been proposed to be phosphorylated by Src, a conundrum exists as to how Src is initially activated (Stover et al 1995, 1996). One potential explanation is that another EGF-R family member (e.g. Erb B2 or Neu), which heterodimerizes with EGF-R, could be responsible for Src activation. For example, Neu can heterodimerize with the EGF-R and become activated after EGF stimulation. Src can bind to activated Neu in vivo, and an interaction between the Src SH2 domain and Neu has been shown in vitro (Muthuswamy & Muller 1995). Thus one possibility is that EGF would induce heterodimerization and activation of Neu and the EGF-R. Src would bind to tyrosine-phosphorylated Neu resulting in activation of Src. Src could then phosphorylate the EGF-R and provide a binding site for additional Src molecules. Determining whether the presence of Neu correlates with Src activation and EGF-R phosphorylation may help to resolve these issues.

FGF RECEPTOR The FGF receptor can also recruit Src PTKs as downstream effectors in some cell types. An increase in Src family kinase autophosphorylation has been observed after FGF treatment of mouse fibroblasts and lung epithelial cells, but no association between Src and the FGF-R has been reported in these cell types (Landgren et al 1995). However, in NIH 3T3 cells, Src PTKs can associate with the FGF-R in vivo, and in vitro binding experiments suggest that this interaction is mediated by the SH2 domain of Src and autophosphorylation sites on the receptor (Zhan et al 1994). In addition, FGF also induces tyrosine phosphorylation of the v-Src substrate, cortactin, and an association between Src and cortactin is also detected after FGF treatment of NIH 3T3 cells (Zhan et al 1993, Schaller et al 1993). Whether Src PTKs are directly responsible for FGF-induced cortactin phosphorylation is unclear, but these studies suggest that Src PTKs also are involved in FGF-R signaling.

INSULIN RECEPTOR While Src PTKs appear to interact directly with RPTKs in the systems described above, a slightly different variation has been observed in insulin receptor signaling. The insulin receptor is composed of an α subunit and two β subunits (Lee & Pilch 1994). The α subunit binds ligand resulting in activation of the β subunits. Activation of the insulin receptor results in tyrosine phosphorylation of a third critical component, an IRS (insulin receptor substrate) protein. The IRS proteins are a family of molecules that have been linked to a variety of receptors including the insulin/IGF-1 receptors and interleukin receptors (White 1994). These proteins contain multiple potential SH2-binding sites and thus have been proposed to act as a link between these

different receptors and SH2-containing effector proteins. One of the proteins shown to interact with IRS-1 after insulin stimulation is the Src PTK, Fyn (Sun et al 1996). As in the cases of the PDGF-R and EGF-R, this interaction is mediated by the SH2 domain of Fyn; however, thus far, no difference in Fyn kinase activity has been observed after insulin treatment. Understanding the precise role of this association in activation of Fyn and the role of Fyn in insulin-receptor signaling are issues to be resolved.

OTHER MECHANISMS OF ACTIVATION Association of Src family kinases with the RPTK or its binding protein represents one mechanism of activation; however, other signaling molecules recruited to the receptor complex could be involved in RPTK-mediated Src activation (Erpel & Courtneidge 1995). For example, the tyrosine phosphatase SHP-2 binds to the PDGF-R and could activate Src PTKs by dephosphorylating the C-terminal negative regulatory tyrosine (Feng & Pawson 1994). EGF induces an increase in H_2O_2 that activates Src PTKs through an unknown mechanisms (Bae et al 1997) (see stress section below). Thus additional mechanisms are likely to function in RPTK-mediated Src activation.

MODIFICATION OF RPTKS BY SRC PTKS As mentioned above for the EGF-R, Src PTKs can also phosphorylate RPTKs. Studies on v-Src-transformed cells suggest that the β subunit of the IGF-1 receptor is a substrate for Src PTKs (Kozma & Weber 1990). In addition, loss of this receptor abrogates the ability of v-Src to induce transformation (Peterson et al 1996). While these studies do not definitively establish a role for the cellular counterpart of v-Src in regulation of RPTKs, tyrosine phosphorylation of RPTKs has been observed in other receptor systems where Src PTKs are activated. For example, Src may phosphorylate the PDGF receptor at a unique site (Hansen et al 1996), and engagement of some G protein–coupled receptors (GPCRs) can activate Src PTKs and induce tyrosine phosphorylation of the EGF-R in a Src-dependent manner (Luttrell et al 1997). Although the role of these phosphorylation events in receptor activation requires further analysis, these studies suggest that a complex regulatory loop exists between Src PTKs and RPTKs (Figure 6).

REQUIREMENT FOR SRC PTKS IN RPTK SIGNALING RPTKs recruit multiple Src PTKs as well as other signaling molecules. Because some of the pathways regulated by these other signaling molecules that interact with RPTKs are functionally redundant with Src PTKs, redundancy can exist at many levels. For example, Src is associated with PI 3-K in PDGF-treated cells; however, PI 3-K can also bind the receptor directly (Kaplan et al 1987, Otsu et al 1991, Escobedo et al 1991). Thus while Src family kinases may be activated by multiple RPTKs, they may not be essential for biological events regulated by the receptor. Studies

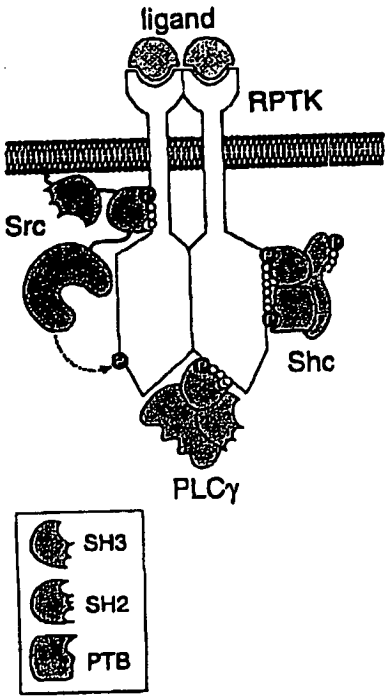

Figure 6 Interaction of RPTKs with Src and several representative SH2-containing signaling proteins. Src interacts with tyrosine phosphorylated motifs in RPTKs through its SH2 domain. Other proteins, such as Shc and PLCγ shown here, also bind to RPTKs through related SH2 domains. Src can also phosphorylate tyrosine residues on the receptor. The position of the Src SH2 binding motif at the juxtamembrane location represents the location of the Src binding sites on the PDGF receptor.

in some systems, however, suggest that Src family kinases do play a critical role in RPTK signaling pathways. For example, use of dominant-interfering mutants and antibodies against Src PTKs has demonstrated a requirement for these kinases in PDGF, CSF-1, EGF, NGF, and FGF signaling (Luttrell et al 1988, Wilson et al 1989, Kremer et al 1991, Twamley-Stein et al 1993, Roche et al 1995b). Although some of the results could be cell-type dependent, these studies indicate that Src PTKs are critical mediators in receptor tyrosine kinase signaling pathways (Figure 6).

G Protein–Coupled Receptors

G protein–coupled receptors (GPCR) form a large family of seven transmembrane-spanning proteins. These receptors are linked to heterotrimeric G protein

Table 4 G protein-coupled receptors linked to Src PTKs

Receptor	Src PTK	Reference
LPA	Src	Luttrell et al 1996
$\alpha 2_A A$	Src	Chen et al 1994, Luttrell et al 1996
Thrombin	Src, Fyn, Yes	Clark & Brugge 1993, Chen et al 1994, Daub et al 1996
M1	Src	Chen et al 1994
Angiotensin II	Src	Marrero et al 1995, Schieffer et al 1996
ET-1	Src	Simonson et al 1996
Bombesin	Src, Fyn, Yes	Rodriguez-Fernandez & Rozengurt 1996
Bradykinin	Src, Fyn, Yes	Rodriguez-Fernandez & Rozengurt 1996
Vasopressin	Src, Fyn, Yes	Rodriguez-Fernandez & Rozengurt 1996
FMLP	Lyn	Torres & Ye 1996
PAF	Src, Lyn, Fyn	Dhar & Shukla 1991, Kuruvilla et al 1994

complexes composed of α, β, and γ subunits (van Biesen et al 1996). Although initial studies suggested that these receptors engaged a set of signaling proteins distinct from those used by other receptor PTKs, increasing evidence suggests that there is great overlap between the effectors of GPCRs and RPTKs. For example, the MAP kinase cascade is activated after engagement of both RPTKs and GPCRs. Similarly, tyrosine phosphorylation also plays a role in certain GPCR signaling pathways. Src PTKs have been implicated in at least 11 different GPCR pathways that include G_i- and G_q-coupled receptors (Table 4). One $G_{\alpha o}$-coupled receptor may also be linked to Src PTKs (Diverse-Pierluissi et al 1997). Evidence for the involvement of Src PTKs in these pathways includes activation of the catalytic activity of one or more Src PTKs, association of these kinases with other signaling proteins, or a requirement for these kinases in certain GPCR-regulated events.

ACTIVATION A two- to threefold activation of Src PTKs has been detected after engagement of multiple GPCRs. For example, endothelin-1 (ET-1) and platelet activating factor (PAF) stimulate the catalytic activity of Src, and thrombin increases the catalytic activity of Src, Fyn, and Yes (Dhar & Shukla 1991, Clark & Brugge 1993, Chen et al 1994, Daub et al 1996). Src shows an increased reactivity to an autophosphorylation-specific antibody after LPA treatment, suggesting that Src is activated in response to LPA (Luttrell et al 1996). Thus engagement of GPCRs can activate Src PTKs.

How GPCRs induce Src activation has not been elucidated. Although binding to the receptor could be a potential mechanism, thus far no direct association between the GPCR and Src has been observed. However, the heterotrimeric G protein subunits are important for Src PTK activation. Overexpression of $G_{\beta\gamma}$ subunits in COS7 cells induces a two- to threefold increase in autophosphorylated

Src, and activation of Src by G_i-coupled receptors is downstream of the $G_{\beta\gamma}$ subunits (Luttrell et al 1997). The activation of Src PTKs by $\alpha 2_A$-adrenergic receptor ($\alpha 2_A$AR) and thrombin is sensitive to pertussis toxin (PTX), which inactivates G_i (Chen et al 1994).

Protein tyrosine phosphatases The above results implicate $G_{\beta\gamma}$ subunits in GPCR-mediated Src PTK activation. Because there is no evidence for a physical interaction between G protein subunits and Src PTKs, other proteins/molecules are needed to link these kinases to GPCRs. Studies on thrombin-, bombesin-, bradykinin-, and vasopressin-mediated activation of Src PTKs have suggested a role for tyrosine phosphatases. Thrombin treatment of platelets induces a transient, but rapid, dephosphorylation of Src, which precedes the increase in catalytic activity (Clark & Brugge 1993). In Swiss 3T3 cells, bombesin-, bradykinin-, and vasopressin-induced activation of Src is blocked by vanadate, a tyrosine phosphatase inhibitor (Rodriguez-Fernandez & Rozengurt 1996). These results suggest that a tyrosine phosphatase lies between the $G_{\beta\gamma}$ subunits and Src. At least two different PTPs have been implicated in signaling downstream of G protein–coupled receptors. In platelets, both PTP1B and SHP1 are activated in response to thrombin treatment (Frangioni et al 1993, Li et al 1994, 1995). Although SHP1 has been proposed to have a negative regulatory role in cytokine signaling, activation of SHP1 occurs very rapidly after thrombin stimulation (Li et al 1994, 1995, Imboden & Koretsky 1995). Thus in platelets, SHP1 could play a role in thrombin-induced Src PTK activation. A second tyrosine phosphatase, PTP1B, is activated by proteolytic cleavage (Frangioni et al 1993). This cleavage is dependent upon integrin engagement, which suggests that it is activated downstream of Src PTKs. PTP1B, therefore, is unlikely to be involved in the initial activation of Src PTKs but could participate in maintaining activation. In fibroblasts, a pertussis toxin-sensitive PTP activity has also been found to copurify with $G_{\alpha o/i}$ subunits, which suggests that PTPs can couple with heterotrimeric G proteins (Dent et al 1996). Identification of this phosphatase activity and additional studies on the role of PTPs in GPCR-mediated signaling should help to resolve whether PTPs are involved in activating Src PTKs.

PI 3-K Inhibitors of PI 3-K can block GPCR-mediated tyrosine phosphorylation, suggesting that PI 3-K is important for activation of Src PTKs (Lopez-Illasaca et al 1997). An isoform of PI 3-K, PI 3-Kγ, can be activated by $G_{\beta\gamma}$ subunits. Because PI 3-Kγ contains a PH domain and because some PH domains can bind to $G_{\beta\gamma}$ subunits, $G_{\beta\gamma}$-mediated activation of PI 3-Kγ may result from binding of $G_{\beta\gamma}$ to PI 3-Kγ (Touhara et al 1994). Regardless of the mechanism of activation of PI 3-K, production of phosphoinositol products of this kinase could be involved in subsequent activation of Src PTKs. One of the products of PI 3-K, PI 3,4,5P, can bind the Src SH2 domain (Rameh et al 1995). Binding of PI 3,4,5P to the Src SH2 domain could displace interaction of the SH2 domain with the negative regulatory tail, leading to Src activation. Thus PI 3-K could play a role in the initial activation of Src PTKs in some GPCR systems. Determining whether PI 3,4,5P can directly activate Src PTKs will help strengthen this model.

FAK/Pyk2 Activation of FAK family kinases could also play a role in GPCR-mediated Src PTK activation. FAK is tyrosine phosphorylated following engagement of many different GPCRs (see below), and the FAK-related kinase Pyk2 also becomes tyrosine phosphorylated after LPA or bradykinin treatment of PC12 cells (Leeb-Lundberg & Song 1991, Sinnett-Smith et al 1993, Rankin et al 1994, Chrzanowska-Wodnicka & Burridge 1994, Polte et al 1994, Rozengurt 1995, Tippmer et al 1996, Dikic et al 1996). As described in the Integrin section, tyrosine phosphorylated Pyk2/FAK interacts with Src via the Src SH2 domain and a high-affinity binding site on Pyk2/FAK. In PC12 cells, LPA and bradykinin induce an association between Src and Pyk2, and Src associated with Pyk2 is activated. In addition, expression of Pyk2 in 293T cells results in Src activation, but this does not occur when a kinase-inactive or autophosphorylation mutant of Pyk2 is expressed (Dikic et al 1996). Thus Src binding to Pyk2/FAK could result in activation of Src. However, phosphorylation of Pyk2 is decreased when Csk is overexpressed, and bombesin can activate Src in the absence of FAK phosphorylation (Rodriguez-Fernandez & Rozengurt 1996, Dikic et al 1996). These results suggest that GPCR-mediated activation of Src occurs by both FAK family-dependent and -independent mechanisms.

RPTKs As indicated above, tyrosine kinases other than Src PTKs appear to play a role in GPCR signaling. Activation of the LPA receptor, $\alpha 2_A$AR or thrombin receptor induces tyrosine phosphorylation of the EGF-R, and angiotensin II can induce tyrosine phosphorylation of the PDGF-R (Linseman et al 1995, Daub et al 1996). Phosphorylation of these receptors occurs through a nonautocrine mechanism, and dominant-interfering mutants of Src PTK or overexpression of a negative regulator of Src PTKs blocks tyrosine phosphorylation of the RPTKs (see below) (Luttrell et al 1997). Thus it is likely that Src PTKs are responsible for regulating tyrosine phosphorylation of the receptor tyrosine kinases. Phosphorylation of these receptors may provide docking sites for downstream signaling proteins involved in transducing the GPCR signal. Further studies are needed to elucidate the link between Src PTKs and RPTKs and understand the role of RPTKs in G protein–coupled receptor signal transduction.

REQUIREMENT FOR SRC PTKs Although the precise role of RPTKs in GPCR-mediated signaling is unclear, a requirement for Src family kinases has been established for some G protein–coupled receptor pathways. Using inhibitory antibodies, dominant-interfering mutants or overexpression of Csk, Src PTKs

appear to be necessary in endothelin-1, LPA, and angiotensin II signaling (Simonson et al 1996, Schieffer et al 1996, Luttrell et al 1997). For example, antibodies to Src block angiotensin II-stimulated tyrosine phosphorylation. Interestingly, antibodies to Fyn or Yes have no effect, which suggests that Src plays a specific role in AT-II signaling (Schieffer et al 1996). In addition, LPA-induced tyrosine phosphorylation of Shc, EGF-R, and MAPK is inhibited by overexpression of Csk or expression of a dominant-interfering mutant of Src (Luttrell et al 1997). Similar studies on ET-1-mediated responses have shown a requirement for Src in *fos* induction (Simonson et al 1996). Thus Src PTKs are necessary for at least some GPCR-mediated events. Identification of downstream targets of these kinases and their role in the biological responses mediated by these receptors (e.g. proliferation or migration) should help to further define the function of Src PTKs in GPCR signal transduction.

Cytokine Receptors

The cytokine receptor superfamily, a large class of receptors, has been subdivided into two groups. This distinction is based on shared structural features of each class. Class I receptors contain an extracellular WSXWS motif (Bazan 1990). Examples include single-chain receptors such as the erythropoietin receptor (Epo-R) and prolactin receptor (Prl-R) and multisubunit receptors such as the interleukin 2 receptor (IL2-R) and granulocyte macrophage colony stimulating factor receptor (GM-CSF-R). The Class II receptor family, which is distinguished in part by its extracellular cysteine pairs, is composed of the antiviral factor receptors (interferon α, β, γ), the IL10-R, tumor necrosis factor receptor (TNFR), and the p75 NGF receptor. Both classes of receptors are capable of activating a signaling cascade that can regulate growth, differentiation, cell survival, and multiple specialized cell functions (Briscoe et al 1994). As in other receptor systems, tyrosine phosphorylation is induced following receptor engagement. The receptor subunits do not contain any intrinsic tyrosine kinase activity; however, at least three cytoplasmic PTK families have been shown to be involved in cytokine superfamily signaling: Jak kinases, SYK/ZAP 70 kinases, and Src PTKs (Ihle et al 1995). Src PTKs participate in signaling cascades initiated by many Class I receptors and at least one Class II receptor (Table 5). The evidence implicating Src PTKs in these receptor pathways includes cytokine-induced activation of Src PTKs, association of Src PTKs with the receptor subunits, cytokine-stimulated association of Src PTKS with other signaling components, and Src family kinase-mediated tyrosine phosphorylation of the receptor subunit(s) or downstream effectors.

IL-2 RECEPTOR One of the first examples of Src PTK involvement in cytokine signaling came from analysis of IL-2 pathways (Briscoe et al 1994, Taniguchi

Table 5 Cytokine receptors coupled to Src PTKs

Receptor	Src PTK	Reference
IL-2	Fyn, Lck, Lyn	Hatakeyama et al 1991, Kobayashi et al 1993, Eljaafari et al 1995
IL-3	Fyn, Hck, Lyn	Kobayashi et al 1993, Anderson & Jorgensen 1995, Yousefi et al 1996
IL-4	Fyn, Lck	Ikizawa et al 1994, Wang et al 1996a
IL-5	Fyn, Lyn	Appleby et al 1995, Yousefi et al 1996
IL-6	Hck	Ernst et al 1994
IL-7	Fyn, Lck, Lyn	Seckinger & Pougereau 1994, Page et al 1995
IL-11	Src, Yes	Yang & Yin 1995, Fuhrer & Yang 1996a, Fuhrer & Yang 1996b
IL-12	Lck	Pignata et al 1995,
IL-15	Lck	Adunyah et al 1997
Prolactin	Fyn, Src	Clevenger & Medaglia 1994
G-CSF	Lyn	Corey et al 1994
GM-CSF	Lyn, Yes, Hck	Corey et al 1994, Linnekin et al 1994, Jucker & Feldman 1995, Yousefi et al 1996
TNF	Fgr	Berton et al 1994, Guy et al 1995
EPO	Src	Kitanaka et al 1994
Oncostatin M	Yes, Fyn, Src	Schieven et al 1992
4.1BB	Lck	Kim et al 1993

1995, Taniguchi et al 1995). IL-2 plays an important role in the proliferation of antigen-stimulated T cells but is also involved in other immune responses. In T cells, IL-2 stimulation results in the activation of the Src PTK, Lck (Horak et al 1991). Activation of Lck correlates with Ser/Thr phosphorylation of Lck, but the precise role of these modifications in Lck activation is unclear. Lck can also bind directly to the β subunit of the IL2-R (Hatakeyama et al 1991). Unlike interactions with other receptors, this interaction is mediated through the N-terminal part of Lck's kinase domain. This region of Lck interacts with an acidic region of the IL-2 receptor β subunit known as the A region. Receptors lacking this region are unable to bind or activate Lck, suggesting that this interaction is important for IL-2-mediated Lck activation. In addition, receptors that have mutations in a second region, the S region, also fail to activate Lck, although these receptors can still bind Lck. Thus the S region may bind a critical regulator of Lck. How Lck activation occurs upon IL-2 stimulation and whether the S region does, in fact, bind an activator of Lck remain to be determined.

Role of Src PTKs in IL-2 signaling Whether Lck is required for IL-2 signaling is uncertain. The mutational studies described above indicate that the A region of the IL-2 receptor β_c subunit (which interacts with Lck) is not

required for IL-2-mediated proliferation. BAF-BO3 cells expressing an IL-2 receptor mutant lacking the A region can still proliferate in response to IL-2 (Miyazaki & Taniguchi 1996). In addition, natural killer (NK) cells derived from Lck-deficient mice can still proliferate in response to IL-2. In contrast to these results, certain T cell clones expressing IL-2R β-receptors with deletions in the A region have defects in their mitogenic response to IL-2 (Miyazaki & Taniguchi 1996). Thus Lck may be required for IL-2 signaling in specific T cell populations. The limited requirement for Lck function in IL-2 signaling could be due to the redundant functions of Src PTKs. Other Src PTKs (e.g. Fyn and Lyn) have been found to interact with the IL-2Rβ subunit in different cells, suggesting that multiple Src PTKs can function in IL-2 signaling pathways (Kobayashi et al 1993).

OTHER CYTOKINE RECEPTORS While the requirement of Src PTKs in IL-2 signaling may be unresolved, Src PTKs have been shown to couple with several other cytokine receptors and to play a critical role in some of these cytokine receptor pathways. For example, studies on IL-5 signaling in B cells has shown that at least two kinases, Lyn and Fyn, are activated (Appleby et al 1995, Yousefi et al 1996). Both kinases can also associate with the β subunit, although the regions important for this association are unclear. Analysis of B cells from *fyn*-deficient mice indicate that loss of *fyn* results in defects in IL-5 signaling (Appleby et al 1995). In K562 cells, expression of anti-sense Src mRNA decreases EPO-induced proliferation and blocks hemoglobin synthesis and glycophorin expression (Kitanaka et al 1994). Where Src fits in the EPO-R signaling cascade, however, has not been determined. Regardless, these studies indicate that Src PTKs can function downstream of the cytokine receptor superfamily and together with other cytoplasmic PTKs form a tyrosine kinase network that mediates effects on growth, differentiation, and cell survival. Additional studies determining how Src PTKs are activated, the precise pathways they regulate, and how they are linked to other cytoplasmic PTKs should provide insight into the function of these kinases in cytokine signal transduction.

GPI-Linked Receptors

A structurally unique class of receptors that couples with Src family kinases are GPI-linked receptors. These receptors are extracellular proteins anchored to the outer leaflet of the plasma membrane via a GPI moiety (Englund 1993). GPI-linked receptors have been best characterized in hematopoietic cells; however, they are expressed in many cell types (Rudd et al 1993). Engagement of these receptors induces a broad spectrum of phenotypic alterations in cells, including migration, neurite extension, and proliferation (Rudd et al 1993, Bohuslav et al 1995, Zisch et al 1995). Several of the GPI-linked receptors have been shown

Table 6 GPI-linked receptors coupled to Src PTKs

Receptor	Src PTK	Reference
Thy-1	Fyn, Lck, Lyn	Stefanova et al 1991, Thomas & Samelson 1992, Narisawa-Saito et al 1996
Ly-6	Lck	Stefanova et al 1991
CD14	Lyn, Hck, Fgr, Lck	Stefanova et al 1991
CD48	Lck	Stefanova et al 1991
CD24	Lck	Stefanova et al 1991
CD55	Fyn, Lck	Shenoy-Scaria et al 1992
CD59	Lck	Stefanova et al 1991
F11	Fyn	Zisch et al 1995
F3	Fyn	Olive et al 1995
uPAR	Fyn, Lyn, Hck, Fgr	Bohuslav et al 1995

to cause an induction of tyrosine phosphorylation and activation of Src family PTKs (see Table 6). The mechanism whereby these receptors couple with PTKs and transduce signals to the intracellular environment is not understood. Because they lack transmembrane and intracellular domains, GPI-linked receptors are incapable of conventional signaling to the cytoplasm. The evidence connecting GPI-linked receptors to Src PTKs and the proposed models for how these proteins couple with each other are discussed below.

CD14 Lipopolysaccharide (LPS) binds to a LPS-binding protein (LBP) and the GPI-linked protein CD14 (Ulevitch & Tobias 1995). LPS treatment of macrophages results in an increase in tyrosine phosphorylation and activation of three Src PTKs: Lyn, Hck, and Fgr (Stefanova et al 1991). The role of Src PTKs in LPS signaling may be dispensable. Although the level of total cell phosphotyrosine is reduced, bone marrow-derived macrophages from *fgr-/-; hck-/-; lyn-/-* mice have no obvious defects in LPS-induced activation (Meng et al 1997).

Thy-1 In T cells, cross-linking of the GPI-linked protein Thy-1 also causes an increase in total cell phosphotyrosine, and at least two Src PTKs, Fyn and Lck, are implicated in this event (Stefanova et al 1991, Thomas & Samelson 1992). Fyn and Lck coprecipitate with Thy-1, and this interaction is dependent on the GPI anchor. In addition, thymocytes from *fyn-/-* mice are defective in Thy-1 signaling, indicating that Fyn plays a critical role in this GPI-linked receptor pathway (Lancki et al 1995).

F3 and F11/contactin Fyn has also been found to associate with F3 and contactin/F11, two other GPI-linked receptors in neurons (Olive et al 1995, Zisch et al 1995). F3 is a member of the immunoglobulin superfamily and is involved

in regulating neurite extension and repulsion. Fyn and the adhesion molecule L1 have been found to complex with F3 in neural tissues (Olive et al 1995). Because L1 is a transmembrane protein, it may be involved in linking Fyn to F3, but additional studies are necessary to address this possibility. Cross-linking of the GPI-linked cell adhesion molecule contactin/F11 also results in the coprecipitation of Fyn. In addition, there is an increase in total cell phosphotyrosine (Zisch et al 1995). Whether Fyn is involved in the biological events regulated by these GPI-linked receptors in vivo remains to be determined.

ASSOCIATION Because Src PTKs are found on the inner face of the plasma membrane and on GPI-linked receptors on the outer leaflet, it is unclear how these molecules are linked to each other. One model proposes that a transmembrane coreceptor links the GPI and Src PTKs. A candidate bridging protein is an 85-kDa transmembrane protein found in Thy-1-Src PTK complexes (Stefanova & Horejsi 1991). A contactin-associated transmembrane protein, p190^{Caspr}, has recently been identified (Peles et al 1997). This protein contains a proline-rich sequence in its cytoplasmic domain that could mediate coupling with Fyn through the Fyn SH3 domain. In the case of the urokinase plasminogen activator receptor (uPA-R), two integrins, LFA-1 and CR3, have been shown to associate with this complex along with Src PTKs (Bohuslav et al 1995). Because Src PTKs have been linked to integrin signaling, it is possible that integrins could bridge GPI-linked receptors to Src family kinases.

A second model, based on studies on two GPI-linked proteins found in T cells, CD59 and CD55, suggests that Src PTKs colocalize with GPI-linked proteins. Colocalization is mediated by their N-terminal fatty acylation modifications (myristylation and palmitylation) (Shenoy Scaria et al 1993, Rodgers et al 1994). Mutations that prevent myristylation or palmitylation of Lck or Fyn abrogate their ability to interact with CD55 and CD59. In addition, Src, which is myristylated but not palmitylated, cannot interact with CD55 or CD59. Replacement of the first 10 amino acids of Src with the corresponding sequences in Fyn or Lck, or mutation of serine residue 3 or 6 in Src to Cys (residues that are palmitylated in Fyn), allows Src to couple with CD55 and CD59. These mutations allow palmitate addition to Src, which appears to be important for targeting Src PTKs to the same membrane subdomain where GPI-linked proteins are found. Thus rather than providing direct interaction, the lipid modification may colocalize Src PTKs and GPI-linked proteins in glycoprotein-rich membrane domains. In support of this hypothesis, Src PTKs and GPI-linked proteins can be dissociated by raising the temperature of the membranes from 4 to 37°C (Rodgers et al 1994). The change in temperature is sufficient to allow triton-solubilization of the membrane fraction containing GPI-linked proteins. This model does not completely rule out the possibility of a coreceptor. Thus both interaction with a coreceptor(s) and localization of Src by lipid modifications may be important for mediating Src PTKs interactions with GPI-linked receptors. In addition, because cross-linking of GPI-linked receptors results in colocalization of numerous signaling molecules (e.g. G protein subunits and integrins), these molecules may also be important for connecting Src PTKs to GPI-linked receptors (Lisanti et al 1994, Bohuslav et al 1995, Solomon et al 1996).

ACTIVATION As indicated above, cross-linking of GPI-linked proteins induces tyrosine phosphorylation of multiple cellular proteins and recruitment of Src PTKs; however, it is uncertain how these kinases are activated upon ligation of the GPI-linked receptors. It is possible that a tyrosine phosphatase may be brought to the GPI-linked receptor complex upon receptor ligation. Alternatively, recruitment of a coreceptor may provide a binding site for SH2 or SH3 domains, which would activate Src PTKs. Identifying potential coreceptors and the role of tyrosine phosphatases in GPI signaling may help to address these questions. It should be noted that in some T cells, glycolipid-enriched membrane (GEM) domains (which contain GPI-linked proteins) have been proposed to sequester Src PTKs from the tyrosine phosphatase CD45, keeping Src PTKs inactive (Rodgers & Rose 1996). Thus movement of SrcPTKs out of these domains could allow access to CD45 and result in their activation. Whether activation of Src PTKs by a GPI-linked receptor such as Thy-1 requires movement of these kinases out of GEM domains has not been determined.

Channels

Channels play important roles in regulating the influx and efflux of small molecules and ions that regulate cellular functions. Several voltage-gated and ligand-gated channels have been shown to couple with Src PTKs including K^+ channels, the IP_3 receptor and other Ca^{2+} channels, and glutamate, NMDA, and *N*-acetylcholine receptors (Zhao et al 1992, Rusanescu et al 1995, Swope et al 1995, Suzuki & Okumura Noji 1995, Calautti et al 1995, Holmes et al 1996, Jayaraman et al 1996, Yu et al 1997). The evidence that Src PTKs physically associate with some of these receptors and, under certain conditions, mediate tyrosine phosphorylation suggests that Src PTKs may regulate channel function. However, Src PTKs can also be activated by these channels, suggesting that Src PTKs or kinases participate in the regulation of cellular functions induced by these receptors. Thus Src kinases may be involved in both upstream and downstream regulation of channel activity.

Voltage-Gated Channels

Ca^{2+} CHANNELS Regulation of intracellular and extracellular calcium levels is important for diverse biological responses including cell proliferation and differentiation. In PC12 cells, depolarization-induced neurite outgrowth requires

the function of a voltage-gated Ca^{2+} channel. Activation of this channel results in an increase in Src-specific activity, and expression of a dominant-interfering mutant of Src blocks depolarization-induced neurite outgrowth (Rusanescu et al 1995).

Primary keratinocytes exposed to Ca^{2+} undergo a differentiation program that includes growth arrest, expression of specific keratins, and formation of desmosomes (Hennings et al 1989, Hennings et al 1992, Dlugosz & Yuspa 1993, 1994). These events are associated with an elevation in the level of total cell phosphotyrosine-containing proteins including the v-Src substrate cortactin (Filvaroff et al 1990, 1994, Calautti et al 1995). In human keratinocytes, Ca^{2+} induces a rapid increase in Src-specific activity and association of Src with three cellular proteins (Zhao et al 1992). Interestingly, Yes-specific activity decreases upon Ca^{2+} treatment (Zhao et al 1993). In the mouse, Fyn has also been proposed to play a role in Ca^{2+}-mediated keratinocyte differentiation (Calautti et al 1995). Analysis of keratinocytes from *fyn*-deficient mice suggests that there is a defect in their differentiation response. These defects include a reduction in expression of specific differentiation markers (e.g. keratin 1 and filaggrin) and loss of cortactin tyrosine phosphorylation. In normal mouse keratinocytes, Ca^{2+}-induced activation of Fyn is observed, but the increase in Fyn-specific activity is only detected after 4 h. These results suggest that in the murine system, Fyn may not be functioning directly downstream of a Ca^{2+} channel. Instead, protein kinase C, which is regulated by intracellular Ca^{2+} concentrations, may be involved in Fyn activation. Treatment of keratinocytes with phorbol ester, an activator of PKC, results in a rapid (2 min) activation of Fyn-specific activity. The FAK-related PTK Pyk2 could also play a role in regulation of Fyn in mouse keratinocytes. Pyk2 is regulated by calcium and can associate with and activate Src PTKs (Lev et al 1995, Dikic et al 1996). Thus although Fyn does play a role in Ca^{2+}-induced differentiation of mouse keratinocytes, Fyn function/activation may not be directly coupled to a Ca^{2+} channel. Instead, downstream effectors like PKC or Pyk2 may be more directly involved in activation of Fyn.

It should be noted that in the keratinocyte system, Ca^{2+} may trigger some responses via effects on other cellular receptors. Ca^{2+}-induced tyrosine phosphorylation in keratinocytes has been linked to a channel; however, extracellular Ca^{2+} can also regulate cell adhesion molecules (Gumbiner 1993, Filvaroff et al 1994). For example, integrins and cadherins require Ca^{2+} for cell-cell and cell-matrix interactions. Because some adhesion molecules have been shown to activate Src PTKs, these receptors could also be involved in Src PTK activation in keratinocytes.

It is not known if, or, indeed, how these kinases are linked to Ca^{2+} channels and what the mechanism of activation might be. In human keratinocytes, where rapid activation of Src is observed, the increase in kinase activity is accompanied by dephosphorylation of Src, suggesting that a tyrosine phosphatase is important for regulating Src function downstream of the Ca^{2+} channel (Zhao et al 1992). In mouse keratinocytes, Ca^{2+} induces dephosphorylation of the negative regulatory tyrosine of Fyn (Calautti et al 1995).

K^+ CHANNELS Studies on voltage-dependent potassium channels suggest that Src PTKs can also regulate ion channel function. In T cells, tyrosine phosphorylation of the Kv1.3 K^+ channel correlates with an inhibition of channel function, and this phosphorylation is blocked in Jurkat cells, which lack Lck (Szabo et al 1996). Src can also associate with the human voltage-dependent potassium channel Kv1.5, in vivo and in vitro via the SH3 domain of Src and a proline-rich sequence in the receptor (Holmes et al 1996). In addition, co-expression of v-Src induces tyrosine phosphorylation of Kv1.5 and a decrease in channel activity. Whether such a modification occurs under normal physiological conditions has not been determined. It is also unclear whether tyrosine phosphorylation per se or binding of the Src SH3 domain with the Kv1.5 channel is responsible for suppression of channel function.

Ligand-Gated Channels

n-AChR A ligand-gated channel, nicotinic acetyl choline receptor (n-AChR), can also interact with Src PTKs. In chromaffin cells, the n-AChR, which stimulates Ca^{2+} influx, is involved in release of catecholamines (Perlman & Chalfie 1977). Both serine/threonine and tyrosine kinases are involved in catecholamine secretion, and Fyn is activated following receptor stimulation (Ely et al 1994, Allen et al 1996). Because activation of Fyn is dependent upon Ca^{2+} influx, the Ca^{2+}-regulated kinase Pyk2 may play a role in the n-AChR-mediated Fyn activation (Lev et al 1995, Cox et al 1996).

Src PTKs also function in n-AChR-mediated membrane depolarization of neuromuscular junctions (Swope et al 1995). Membrane depolarization at neuromuscular junctions involves clustering of the n-AChR in response to neural proteoglycans such as agrin. Studies in the Torpedo electric organ have shown that the δ subunit of the AChR is tyrosine phosphorylated and associates with Fyn and a Fyn/Yes related kinase (Fyk) in response to agrin-induced clustering. This association is likely to be mediated by the SH2 domain because a Fyn or Fyk SH2 domain fusion binds the phosphorylated δ receptor subunit. In mammalian cells, the role of Src PTKs in n-AChR signaling is unclear. Tyrosine phosphorylation of the β, and δ subunits has been observed; however, it is unclear if Src PTKs are associated with the receptor (Wallace et al 1991, Qu et al 1994). Studies in mice have implicated a third tyrosine kinase, MuSK, in tyrosine phosphorylation of the AChR (DeChiara et al 1996, Glass et al 1996).

MuSK is a RPTK that is complexed with an accessory protein found in myotubes. This complex can bind agrin, which results in the activation of MuSK and clustering of the AChR. Loss of MuSK or agrin prevents AChR clustering, which suggests that both proteins are required. In addition, MuSK activation correlates with phosphorylation of the β subunit of the AChR, and this phosphorylation is important for AChR aggregation. Because Src PTKs can couple to RPTKs, it is possible that Src PTKs may interact with MuSK and phosphorylate the AChR. *src-/-;fyn-/-* mice show no defects in agrin-induced clustering, suggesting that these two Src PTKs have either a redundant role or no role in ACHR clustering (S Burden, personal communication).

1,4,5 INOSITOL TRISPHOSPHATE (IP_3) RECEPTOR/Ca^{2+} CHANNEL Another example of a channel that may couple to Src PTKs is found in T cells. Engagement of the TCR induces multiple cellular responses including increases in tyrosine phosphorylation and alterations in Ca^{2+} levels. Changes in Ca^{2+} concentration partly result from production of the second messenger IP_3. IP_3 binds to a receptor located on the endoplasmic reticulum (IP_3-R) (Harnick et al 1995). The IP_3-R is a ligand-gated calcium channel that, when activated, releases intracellular calcium. In T cells, stimulation of the TCR induces a physical association between the IP_3-R and Fyn and tyrosine phosphorylation of the receptor (Jayaraman et al 1996). Thymocytes from *fyn*-deficient mice have a reduction in IP_3-R tyrosine phosphorylation, which correlates with a defect in TCR-mediated Ca^{2+} release. Thus it is likely that Fyn may function in vivo to regulate this ligand-gated Ca^{2+} channel.

NMDA RECEPTOR The NMDA receptor, a ligand-gated channel, plays an important role in neuroplasticity and synaptic transmission in the central nervous system (Hollmann & Heinemann 1994). Both serine/threonine and tyrosine kinases have been implicated in NMDA signaling pathways (Raymond et al 1993, Wang & Salter 1994, Wang et al 1996b). Two other Src family kinases, Src and Fyn, have been found to function downstream of the NMDA receptor (Grant et al 1992, Yu et al 1997). Src can coprecipitate with the NMDA receptor, and activation of Src PTKs by incubation with a high-affinity ligand for the SH2 domain results in an increase in channel activity (Yu et al 1997). This change in channel activity is dependent upon sequences in the unique domain of Src, and an antibody recognizing the unique domain of Src decreases NMDA channel gating. Thus Src interacts with the NMDA receptor or an associated protein through its unique domain, and this interaction is important for regulation of the receptor by Src. Since at least two of the receptor subunits are tyrosine phosphorylated, it is possible that the NMDA receptor may be a substrate of Src. Alternatively, a second Src PTK, Fyn might be involved in phosphorylating the receptor. Fyn has also been found to phosphorylate the NMDA receptor in vitro and has been implicated in long-term potentiation (LTP), a process regulated by the NMDA receptor (Grant et al 1992, Grant & Silva 1994, Suzuki & Okumura Noji 1995). How Src PTKs are activated by the NMDA receptor is unclear, but these kinases appear to play a role in regulating this ligand-gated channel.

GAP JUNCTIONS Gap junctions are specialized membrane structures that serve as intercellular channels to regulate cell:cell communication. Phosphorylation of the subunits of these junctions, referred to as connexins, regulates gap junctional communication (review, Lau et al 1996). Connexin43 is phosphorylated on tyrosine in v-src transformed cells, and this event correlates with a decrease in gap junction communication (Crow et al 1990, Filson et al 1990, Swenson et al 1990).

Although the role of c-Src in regulation of gap junctions by receptor pathways has not been determined, c-Src can phosphorylate connexin 43 in vitro on sites identical to those observed in vivo (Loo et al 1995). In addition, the cytoplasmic regions of connexin 43 contain proline-rich motifs and tyrosine phosphorylation sites that could provide docking sites for Src PTKs. Thus gap junctions may also be linked to Src PTKs. Although EGF and FGF treatment causes a reduction in junctional communication, connexin 43 is phosphorylated on serine, not tyrosine (Lau et al 1992, Doble et al 1996).

Although additional studies are needed to better define the link between Src PTKs and various channels, the above examples demonstrate that Src family kinases are likely to be involved in relaying signals to and from this class of receptors.

Stress Responses

Stress-inducing agents such as ultraviolet C (UVC) irradiation, heat, or hypoxia trigger signaling pathways that mediate either protection or killing of affected cells. The best-characterized cellular stress responses are those involved in the induction of changes in gene expression. For example, irradiation with short-wavelength ultraviolet light (UVC) induces activation of MAP kinases that activate pre-existing transcription factors (AP-1, TCF/elk-1, NF-κB), which mediate transcriptional activation of *c-fos*, *c-jun*, and other genes (Stein et al 1989, Rahmsdorf et al 1992, Devarey et al 1992, 1993, Radler-Pohl et al 1993). Ras and Raf are activated in this response and dominant-negative variants of these proteins block transcriptional activation (Devarey et al 1992, 1993, Radler-Pohl et al 1993). In addition, UVC treatment leads to induction of dimerization, tyrosine phosphorylation, and internalization of the EGF-R, and a truncated dominant-negative variant of the EGF-R blocks the UVC transcriptional response (Warmuth et al 1994, Miller et al 1994, Sachsenmaier et al 1994, Coffer et al 1995, Huang et al 1996).

c-Src has been implicated in the UVC transcriptional responses by the evidence that c-Src kinase activity is elevated after UVC treatment, and expression of kinase-inactive Src variants inhibits UVC-induced activation of Jun and NF-κB (Devarey et al 1992, 1993). Because Src phosphorylates the EGF receptor (Wasilenko et al 1991) and is associated with, and activated by, the EGF receptor (Oude Weernink et al 1994, Sato et al 1995a), it is possible that Src could act upstream or downstream of the EGF receptor in this response pathway. Likewise, Src could either directly mediate activation of Ras and Raf, or their activation could be dependent on EGF receptor phosphorylation.

EGF-R activation by UVC irradiation can be blocked by antioxidants, and the activation of EGF-R can be mimicked with H_2O_2, suggesting that reactive oxygen intermediates may be responsible for the activation of EGF-R following UVC irradiation (Huang et al 1996). Src family kinases have also been reported to be activated by H_2O_2 and other oxidants (Nakamura et al 1993, Hardwick & Sefton 1995, Gonzalez-Rubio et al 1996). For example, Lck is activated by H_2O_2 in T cells and fibroblasts. Lck activation is not associated with decreased phosphorylation on the C-terminal negative regulatory Tyr505; however, it is dependent on phosphorylation of the catalytic cleft activation loop tyrosine residue analogous to Tyr416 of Src (Hardwick & Sefton 1995). Hardwick & Sefton found that a kinase-inactive mutant variant of Lck is still phosphorylated following H_2O_2 treatment, and they proposed that another tyrosine kinase activated by H_2O_2 treatment may phosphorylate Lck on Tyr394 and activate its catalytic activity (Hardwick & Sefton 1995). Alternatively, H_2O_2 may inhibit a tyrosine phosphatase that constitutively dephosphorylates Y394. Src kinase activation by H_2O_2 treatment has not been detected in all cell types. Activation of Fyn, Lyn, and Lck was not detected in Ramos B cells exposed to H_2O_2 treatment under conditions where Syk was detectably activated (Schieven et al 1993). These results raise the possibility that oxygen intermediates activate Src kinases, which in turn phosphorylate the EGF receptor, leading to activation of Shc, Ras, and Raf. This pathway would mimic the proposed Src-mediated activation of EGF receptor by $G_{\beta\gamma}$-induced signaling pathways (Luttrell et al 1997).

Oxygen deprivation, or hypoxia, also induces changes in gene expression including an induction of the angiogenesis factor, vEGF, as well as endothelin, and PDGF B-chain (Kourembanas et al 1990, 1991). Treatment of cultured cells under hypoxic conditions causes an activation of Src and Fyn kinase activity, and kinase-inactive variants of Src block hypoxia-induced changes in vEGF mRNA accumulation (Mukhopadhyay et al 1995, Seko et al 1996a,b). These results suggest that Src may be involved in promoting angiogenesis in tumors by participating in the induction of vEGF; however, the mechanism for Src activation under these conditions has not been elucidated.

In PC12 cells, activation of stress pathways by sorbitol leads to activation of the FAK-related protein tyrosine kinase, Pyk2/RAFTK/CAKβ. Overexpression of Pyk2 induces Jun-kinase (JNK) activation and a dominant-negative mutant of Pyk2 blocks stress-induced JNK activation (Tokiwa et al 1996). Because Src has been shown to couple with Pyk2 in other PC12 receptor pathways (Dikic et al 1996), it is possible that these two tyrosine kinases may cooperate in stress response pathways in certain cell types.

In summary, a variety of different stress conditions lead to activation of Src kinases. Changes in gene expression mediated by UVC treatment and hypoxia appear to be dependent on Src kinases; however, the precise mechanism that mediates Src activation is not understood, and the downstream effectors of Src activity involved in this response have not been definitively identified.

CELLULAR EVENTS REGULATED BY SRC KINASES

The preceding sections have discussed the activation of Src family kinases by diverse families of receptors that induce cellular responses that affect growth control, survival and differentiation, cytoskeletal arrangements, secretion, channel function and other biological activities. Many of the responses to a specific receptor overlap with those of unrelated receptors (e.g. induction of DNA synthesis), whereas other responses are relatively receptor-specific (e.g. neurite outgrowth). What receptor-mediated events are regulated by Src kinases, and do Src kinases mediate similar or distinct biological events when activated by different receptor classes? In this section, we address these questions by discussing potential effector functions of Src in these receptor pathways.

It is difficult to establish the precise functional roles of individual Src kinases in receptor pathways for many reasons: 1. Closely related kinases appear to play redundant roles in receptor pathways (e.g. the activation of Src, Fyn, and Yes by PDGF). 2. Expression of kinase-inactive, dominant-interfering variants of individual Src family members interferes with the function of multiple Src PTKs owing to the high degree of homology of the SH2 and SH3 domains of these kinases. 3. Src kinases can activate other families of protein tyrosine kinases (e.g. Syk/ZAP, Btk, RPTKs). Therefore, strategies to inhibit Src can block phosphorylation of substrates of downstream kinases. 4. It is difficult to directly correlate functions of mutant, constitutively activated kinases with those of their transiently activated cellular homologues. 5. The dependence of any one receptor on Src PTKs can vary in different cellular environments depending on whether redundant pathways can activate the same biological activity or on how strongly the receptor activates a Src PTK. 6. Small-molecule inhibitors that block the activity of specific PTKs have not been developed.

Src Kinase Substrates

An obvious approach to exploring cellular events regulated by Src PTKs is to identify substrates specifically phosphorylated by these enzymes following

Table 7 v-Src substrates phosphorylated after stimulation of cellular receptors

Substrates	Receptors
Cytoskeletal proteins	
Focal adhesion proteins	
FAK	Integrins, PDGF-R, insulin, NGF-R, EGF-R, FcεRI, bombesin-R, thrombin-R, bradykinin-R, endothelin-R, LPA-R, AngII-R, PAF-R, CCK-R, FMLP-R, gastrin-R
paxillin	PDGF-R, insulin-R, NGF-R, bombesin-R, PAF-R, ICAM, bradykinin-R, FMLP-R, gastrin-R, endothelin-R, LPA-R, AngII-R, CCK-R, TNFR-R, IL-3-R
$p130^{cas}$	Integrins, NGF-R, bradykinin-R, IL-8-R
talin	Thrombin-R, PDGF-R
tensin	Integrins
ezrin	Integrin, Met/HGF-R, CD4, CD3
Other actin cytoskeletal proteins	
cortactin, HS1	FGF-R, integrins, thrombin-R, calcium, TCR, ICAM
AFAP 110	
ezrin	Integrin, Met/HGF-R, CD4, TCR/CD3
Other structural proteins	
catenins (β, γ and p120)	CSF-1R, EGF-R, PDGF-R, Met/HGF-R
connexin 43	PDGF-R
caveolin	Insulin-R
calpactin	PDGF-R, EGF-R, insulin-R
tubulin	TCR, NPG-R
Enzymes	
Other protein tyrosine kinases	
Syk, /ZAP	IRRs, integrins, muscarinic-R, IL-2-R
Tec kinases	IRRs
FAK	See above
Protein serine/threonine kinase	PDGF-R, EGFR, FcεRI, substance P-R
PKCδ	
Enzymes involved in phospholipid metabolism	
PLC-γ,	NGF-R, PDGF-R, EGF-R
p85PI 3-K	Many RPTKs, thrombin-R, most IRRs, integrins, many class I cytokine receptors (not shown to be tyr phos in all these)
SHIP	FcγRI, BCR, m-CSF-R
smGTP-regulatory enzymes	
$p190^{RhoGAP}$	Many RPTKs, FcγRIIIA, BCR, TCR, receptors for bombesin, bradykinin, GM-CSF
$p120^{rasGAP}$	Many RPTKs, BCR, TCR

(*continued*)

Table 7 (*continued*)

Substrates	Receptors
Phosphatases	
SHP-1	Thrombin-R, NGF-R, insulin-R
SHP-2	PDGF-R, EGF-R, EPO-R, prolactin-R
PP2A	EGF-R
Adaptors	
Shc	Most RPTKs, many class I cytokine receptors, most IRRs, CD4, G_iPCRs
DOK (p62 GAP protein)	Many RPTKs, TCR, BCR, CD-2, high calcium
Cbl	TCR, BCR, EGF-R, FcRs
Receptors	
IRRs (ITAMs)	IRRs, CD2
Receptor PTKs	RPTKs, N-CAM, N-cadherin, G_iPCRs

receptor activation. The identification of candidate Src PTK substrates has been facilitated greatly by the analysis of constitutively activated variants of these kinases such as the v-Src transforming protein of RSV. Many candidate v-Src substrates have been identified by comparing the profiles of proteins phosphorylated on tyrosine in control and RSV-transformed cells. Table 7 shows some of the v-Src substrates that have been identified and a partial list of cellular receptors that stimulate tyrosine phosphorylation of these proteins. It is clear that many classes of receptors induce phosphorylation of proteins that are phosphorylated in v-Src-transformed cells, raising the possiblity that Src PTKs are involved in receptor-induced phosphorylation of these proteins. However, it is difficult to establish whether Src kinases are responsible for phosphorylation of these substrates following receptor activation (since multiple PTKs are activated by most receptor pathways) and to determine which cellular responses are regulated by each substrate phosphorylation event.

The properties of these substrates have been discussed extensively elsewhere (Kellie et al 1991, Schaller et al 1993, Brown & Cooper 1996). Since this review is focused on the biological events regulated by Src family kinases, we discuss these substrates only in the context of how they may be involved in specific receptor-induced events postulated to be regulated by Src kinases. Many of the substrates of Src PTKs are involved in the regulation of multiple biological activities. For example, PI 3-K has been implicated in the regulation of DNA synthesis, cell survival, differentiation, lamellipodia formation, chemotaxis and migration, adhesion, and neurite outgrowth (Vanhaesebroeck et al 1996). Likewise, phosphorylation of Shc and activation of the Ras-MAP kinase (MAPK) pathway are involved in induction of DNA synthesis, migration, oocyte maturation, mitosis, and neurite outgrowth. Thus a discussion of Src PTK effectors

involved in biological activities regulated by these kinases is complicated by the pleiotropic activities of Src, as well as many of its substrates.

It is noteworthy that recent studies of the function of Src in integrin signaling and in osteoclasts strongly suggest that the noncatalytic domains of Src also have important effector functions (Kaplan et al 1995, Schlaepfer et al 1997; P Schwartzberg, L Xing, B Boyce & HE Varmus, unpublished results). These studies indicate that the SH2- and SH3-binding domains of Src kinases (and possibly the unique domain) may serve adaptor-type functions independent of catalytic activity.

Adhesion and Spreading

The role of Src in adhesion mediated by integrin receptors for the extracellular matrix protein fibronectin has been examined in *src-/-* fibroblasts. These fibroblasts display a reduced rate of adhesion and spreading on a fibronectin matrix, suggesting that Src is required for optimal adhesion efficiency but is not essential for these events (Kaplan et al 1995). *src-/-* fibroblasts could not be distinguished from Src-expressing fibroblasts on a collagen matrix, indicating that Src may function specifically in fibronectin receptor adhesive responses.

Overexpression of Csk has also been used as a strategy to examine the role of Src in cell adhesion. Overexpression of Csk in HeLa cells causes a conversion of these cells to a spherical, loosely adhered morphology and changes the morphology of $\alpha v\beta 5$ integrin structures on the ventral cell surface. This activity was dependent on the catalytic activity of Csk. Because Csk overexpression can inactivate c-Src by maintaining a high stoichiometry of Y527 phosphorylation, these data provide additional supportive evidence for a role of Src in cell adhesion and spreading. The role of Src in regulating cell adhesion is not known. However, Src catalytic activity does not appear to be critical for this event because the defect in cell adhesion in *src-/-* fibroblasts can be rescued with Src mutants lacking the catalytic domain (Kaplan et al 1995). Rescue requires an intact SH2 and SH3 domain. These results suggest that Src may serve as an adaptor protein to localize specific proteins to adhesive structures (possibly focal adhesions) involved in cell adhesion and spreading.

Focal Adhesion Formation/Disassembly

Focal adhesions and related structures are induced following engagement of many integrins. These structures are specialized membrane-attachment plaques where integrins couple the extracellular matrix with bundled actin cable filaments and complex assemblies of other cytoskeletal proteins (Jockusch et al 1995, Brown & Cooper 1996, Burridge & Chrzanowska-Wodnicka 1996). Focal adhesions are important for cell adhesion, morphology, and cell migration. Fyn

and Yes are also expressed in fibroblasts and may serve redundant functions. The evidence that many v-Src substrates, including paxillin, $p130^{cas}$, talin, vinculin, tensin, FAK, and $\beta 1$ integrin subunit are associated with focal adhesion sites and phosphorylated on tyrosine suggests that Src family kinases may be involved in focal adhesion dynamics (Bockholt & Burridge 1993, Jockusch et al 1995, Schwartz et al 1995, Clark & Brugge 1995, Petch et al 1995, Bhattacharya et al 1995, Brown & Cooper 1996, Burridge & Chrzanowska-Wodnicka 1996).

Tyrosine phosphorylation appears to be involved in the formation of focal adhesions because PTK inhibitors block this event; however, since multiple tyrosine kinases are associated with focal adhesions (Src, Fyn, FAK, Abl), it is not known which kinases are critically involved (Burridge et al 1992, Romer et al 1992, 1994, Seckl & Rozengurt 1993, Ridley & Hall 1994, Chrzanowska-Wodnicka & Burridge 1994, Lewis et al 1996). It is likely that the major role for tyrosine phosphorylation in focal adhesion formation is the induction of protein interactions through creation of SH2-binding sites. However, tyrosine phosphorylation may also regulate the activity of enzymes involved in focal contact assemblies.

The role of Src PTKs in focal adhesion assembly/disassembly is not understood. Src-/- fibroblasts are able to assemble focal adhesions, indicating that Src does not serve a unique function in focal adhesion formation. Fibronectin-induced tyrosine phosphorylation of $p130^{cas}$ is defective in fibroblasts from Src-deficient mouse embryos (Bockholt & Burridge 1995, Hamasaki et al 1996, Vuori et al 1996, Schlaepfer et al 1997). In contrast, cells deficient in FAK, Fyn, or Abl are not defective in phosphorylation of $p130^{cas}$ (Bockholt & Burridge 1995, Hamasaki et al 1996). Because $p130^{cas}$ does not localize to focal adhesions in Src-/- fibroblasts and because kinase-dead Src variants can rescue $p130^{cas}$ localization to focal adhesions and tyrosine phosphorylation, it appears that Src is involved in the recruitment of $p130^{cas}$ to focal adhesions, rather than being directly responsible for its phosphorylation (Schlaepfer et al 1997; K Vuori, unpublished results).

As described above, Src and Fyn bind to FAK and may participate in FAK-mediated signaling events by phosphorylating FAK (thus creating sites for recruitment of proteins to FAK complexes) and, possibly, by regulating FAK kinase activity (Schaller et al 1994, Cobb et al 1994, Xing et al 1994, Eide et al 1995, Calalb et al 1995, 1996, Schlaepfer & Hunter 1996). The role of FAK in focal adhesion assembly/disassembly is not definitively established; however, several lines of evidence indicate that FAK plays an important role in cell migration. It has been postulated that the defect in cell migration in FAK-/- fibroblasts is the result of a defect in disassembly of focal adhesions.

Structures similar to focal adhesions are assembled by integrins in other cell types. Src, Yes, talin, vinculin, spectrin, and other focal adhesion proteins

are associated with large, detergent-insoluble complexes involved in $\alpha_{IIb}\beta_3$-mediated platelet aggregation (Horvath et al 1991, Fox et al 1993). Src-deficient platelets do not show any detectable defects in platelet aggregation; however other Src related kinases may compensate for Src since Yes, Fyn, and Lyn are also recruited to integrin complexes in aggregated platelets (Fox et al 1993).

Lamellipodia

Sheet-like protrusions of actin filaments assembled in a meshwork are referred to as lamellipodia or membrane ruffles. v-Src induces a rapid induction of membrane ruffling within minutes after it is activated [using conditional mutants of v-Src (Boschek et al 1981)]. The substrates involved in these microfilament reorganizations are not known; however, candidate effector proteins include PI 3-K, ezrin, vinculin, cortactin, p190 Rho^{GAP}, and AFAP, which are phosphorylated on tyrosine following stimulation by many classes of receptors (Table 7). The involvement of c-Src or other family members in receptor-induced lamellipodia or other changes in cortical actin-associated structures is not well established. However, Src kinases and several substrates have been implicated in these events by the evidence described below.

PI 3-K regulates the small GTP-binding protein Rac, a potent inducer of membane ruffling (Kotani et al 1994, Wennstrom et al 1994, Nishiyama et al 1994, Ridley et al 1995). Membrane ruffling induced by insulin, IGF-1, scatter factor/HGF, and PDGF is blocked by inhibitors of PI 3-K. Because activated variants of Src activate PI 3-K activity, it is possible that Src-mediated activation of PI 3-K is involved in membrane ruffling induced by certain classes of cellular receptors.

$p190^{RhoGAP}$ is phosphorylated in v-Src-transformed cells and growth factor-stimulated cells (Ellis et al 1990, Bouton et al 1991, Settleman et al 1992). This protein associates with $p120^{RasGAP}$ through $p120^{RasGAP}$ SH2 domains and displays Rho and Rac GTPase-activating activity (McGlade et al 1993, Ridley et al 1993, Chang et al 1995). Its potential involvement in Src-mediated actin rearrangements was highlighted by the evidence that actin stress fiber dissolution and EGF-induced condensation of p190 and $p120^{RasGAP}$ into cytoplasmic, arc-like structures (which delimit regions of greatest stress fiber dissolution) was inhibited in cells overexpressing a kinase-inactive form of Src (Chang et al 1995). These effects correlated with decreased phosphorylation of $p190^{RhoGAP}$. Conversely, overexpression of kinase-active Src increased the rate of appearance and number of cells exhibiting EGF-induced arcs as well as tyrosine phosphorylation of $p190^{RhoGAP}$ (Chang et al 1995). These results suggest the possibility that p190 phosphorylation by Src following EGF receptor activation activates Rho GAP activity of p190, which leads to the early dissolution of actin filaments.

Cortactin is a 80–85 kDa F-actin cross-linking protein enriched in cortical structures such as ruffles and lamellipodia (Wu & Parsons 1993, Huang et al 1997). It is phosphorylated in v-Src-transformed cells, as well as in cells treated with various growth factors, thrombin, or with integrin or ICAM ligands (Table 7) (Maa et al 1992, S Wong et al 1992, Fox et al 1993, Wu & Parsons 1993, Durieu-Trautmann et al 1994, Zhan et al 1994, Vuori & Ruoslahti 1995, Ozawa et al 1995, Bhattacharya et al 1995, Calautti et al 1995). Cortactin redistributes with actin into podosomes after phosphorylation in Src-transformed cells; however, the function of cortactin and the effects of tyrosine phosphorylation are not understood (Wu & Parsons 1993, Okamura & Resh 1995). In vitro phosphorylation of cortactin by c-Src causes a dramatic inhibition of its F-actin cross-linking activity and moderate inhibition of F-actin binding (Huang et al 1997). Studies of cells from *csk-/-* mice suggest that cortactin may be a preferred substrate of Src (relative to Fyn) and that there is a correlation between stress fiber dissolution and cortactin hyperphosphorylation (Thomas et al 1995). In *csk-/-* or *csk-/-; fyn-/-* fibroblasts, cortactin is hyperphosphorylated and localized to podosomes and actin stress fibers are lost. However, cortactin is not hyperphosphorylated in *csk-/-; src-/-* fibroblasts, its localization resembles wild-type fibroblasts, and stress fibers are partially intact.

FGF receptor-induced tyrosine phosphorylation of cortactin takes place 4–8 h after treatment (at the mid-late G1 phase of the cell cycle). This event is coincident with Src activation, and Src coprecipitates with cortactin, suggesting that Src may be responsible for FGF-induced cortactin phosphorylation (Zhan et al 1993, 1994).

Recent studies of the pathogenic bacteria *Shigella flexneri* suggest an involvement of Src and cortactin in actin-cytoskeletal rearrangements that are involved in bacterial invasion (Dehio et al 1995). Invasion of cells by *S. flexneri* occurs via bacterium-directed phagocytosis, which involves actin polymerization at the bacterial entry site. Src is recruited to the entry structure and to the periphery of the phagosome. Bacterial invasion is associated with increased levels of cortactin phosphorylation, which is enhanced in cells overexpressing c-Src. Cortactin is highly enriched in the entry structures and in the periphery of the phagosome.

Ezrin is a widespread protein localized to an actin-containing membrane skeleton. It is a member of the ERM (ezrin/radixin/moesin) family of proteins, which are capable of mediating membrane-cytoskeletal interactions through their amino-terminal talin/band 4.1 domain and their C-terminal actin-binding domain (Tsukita et al 1997, Gould et al 1989, Turunen 1989, Algrain et al 1993). The actin-binding site of ezrin is masked in the intact protein and is believed to be uncovered by conformational changes that relieve intramolecular interactions between the N- and C-terminal halves of ezrin. v-Src phosphorylates ezrin on

two tryrosine residues located in each of the domains of ezrin (Krieg & Hunter 1992). It is possible that tyrosine phosphorylation may affect the intramolecular interactions of ezrin or interactions of ezrin with actin or the plasma membrane. Overexpression of the C-terminal portion of ezrin leads to the formation of lamellipodia (Martin et al 1995), and ezrin antisense oligonucleotides decrease filopodia and lamellipodia formation (Takeuchi 1994).

Ezrin localizes to membrane ruffles induced by EGF (Bretscher 1989). Ezrin is phosphorylated on tyrosine after engagement of scatter factor (Met) receptor PTK, EGF (Bretscher 1989), and cross-linking of either CD4 or CD3 (Bretscher 1989, Thuillier et al 1994, Jiang et al 1995). Although Src family kinases are activated by all these receptors, the involvment of Src family kinases in ezrin phosphorylation was investigated in only one study. Ezrin phosphorylation induced by cross-linking of CD4 was blocked by mutations in CD4 that prevent interaction with Lck (Thuillier et al 1994).

Taken together, these studies provide substantial support for the possibility that Src is involved in receptor-mediated phosphorylation of cytoskeletal proteins that affect cortical actin cytoskeletal rearrangements; however, the precise effects of phosphorylation on these proteins and their role in cytoskeletal rearrangements remain to be determined.

Migration

Cell migration is induced following treatment with a variety of agonists that mediate movement of cells in tissue development, wound healing, lymphocyte homing, tumor metastasis, and other cellular functions. Many signaling molecules can influence cell migration through effects on cell adhesion (affecting either affinity/avidity for adhesive ligands and/or cytoplasmic stabilization of adhesion), cytoskeletal rearrangements involved in dissociation and spreading, or expression of adhesion receptors or signaling molecules. Preliminary findings in several experimental systems suggest that Src may be involved in cell migration. v-Src has been shown to induce migration of PC12 pheochromocytoma cells in combination with PMA (similar to the way NGF and PMA induce migration) (Altun-Gultekin & Wagner 1996).

Fibroblasts from *src-/-* mice display a significantly slower rate of locomotion than wild-type fibroblasts, and this defect is rescued by expression of a kinase-active, but not inactive c-Src variant (Hall et al 1996). In addition, expression of a dominant-negative Src inhibits locomotion that is mediated by the RHAMM-receptor for hyaluronan, and c-Src associates with RHAMM (Hall et al 1996).

c-Src is also implicated in migration induced by EGF in a rat carcinoma cell line. c-Src is activated when NBT-II rat carcinoma cells are treated with EGF when subconfluent (Rodier et al 1995). Under these conditions, the cells undergo epithelial-to-mesenchyme transition, dissociate from cell clusters, and become motile. Cell dissociation and scattering induced by EGF is inhibited following microinjection of kinase-inactive Src, and overexpression of c-Src causes a subpopulation of cells to undergo spontaneous cell dissociation and to display increased sensitivity to EGF. c-Src has also been implicated in endothelial cell migration induced by inhibition of angiotensin II and by the associated stimulation of u-PAR activity (Bell et al 1992). Lastly, migration of T cells induced by lymphocyte chemoattractant factor (LCF) requires CD4 and Lck (Ryan et al 1995). It is of interest that the catalytic activity of Lck is not required for this response. Therefore, CD4-Lck is required for LCF-induced motility; however, this response is independent of the enzymatic activity of Lck. The events downstream of CD4-Lck coupling have not been identified. Src could be acting at many differents steps in the pathways leading to cell migration, possibly through effects on integrin-mediated adhesion (potentially involving FAK), rearrangements of cortical actin-cytoskeleton involved in cell spreading, or changes in gene expression that affect expression of integrins, other adhesive receptors, or downstream signaling molecules.

MAPK has recently been shown to be essential for integrin-induced cell migration. Because Src has been implicated in integrin-induced MAPK activation, it is possible that the role of Src PTKs in cell migration is partially mediated by activation of MAPK (Schlaepfer et al 1997, Klemke et al 1997). In addition, FAK has also been shown to be involved in integrin-mediated cell migration. Overexpression of FAK stimulates cell migration, and this process is defective in cells lacking FAK or expressing FAK-dominant inhibitory variants (Wilson et al 1995, Ilic et al 1995, 1996, Cary et al 1996, Gilmore & Romer 1996). FAK mutants lacking the Src-binding site Y397 do not enhance cell migration when overexpressed in fibroblasts, suggesting that Src, Fyn, or another protein that binds to this phosphorylation site (e.g. PI 3-K) are required for FAK's role in cell migration (Cary et al 1996, Chen et al 1996). Lastly phosphorylation of PI 3-K by Src could also be involved in Src-mediated migration because PI 3-K appears to be involved in regulating cell migration (Bornfeldt et al 1995, Yokote et al 1996).

Cell Cycle Progression

INDUCTION OF DNA SYNTHESIS The ability of constitutively activated forms of Src family members to induce DNA synthesis and cell proliferation implies that activation of the wild-type kinases can stimulate pathways leading to cell proliferation. However, constitutive activation of Src kinases by mutations does not necesssarily mimic activation mediated by receptor-stimulated pathways where these kinases are generally activated only transiently. Therefore, one cannot conclude that activation of Src kinases by cellular receptors is sufficient

to stimulate cell proliferation. More likely, Src kinases contribute to a whole program of events that can lead to stimulation of DNA synthesis and cell cycle progression under natural, receptor-driven activation.

Src family kinases appear to be required for induction of DNA synthesis by several growth factor receptor protein tyrosine kinases. Microinjection of DNA encoding a kinase-inactive form of Src or Fyn (Twamley-Stein et al 1993) or a neutralizing antibody that recognizes a conserved sequence in the C-proximal tail of Src, Fyn, and Yes inhibits induction of DNA synthesis by EGF, PDGF, and CSF-1. In addition, expression of a kinase-inactive or kinase domain-deleted Src mutant in *src-/-* fibroblasts blocks PDGF induction of DNA synthesis (Broome & Hunter 1996). The SH2 domain of Src is required for association with the PDGF receptor; however, the SH3 domain appears to play an important role in PDGF- and EGF-induced DNA synthesis because mutant forms of Src carrying mutations in the SH3 domain dominantly interfere with the induction of DNA synthesis by these growth factors (Broome & Hunter 1996, Erpel et al 1996).

Recent evidence suggests that the induction of *myc* transcription is the PDGF-β pathway dependent on Src/Fyn/Yes (Barone & Courtneidge 1995). PDGF induction of *myc* is blocked by Src-inhibitory antibodies, and the antibody-induced block in DNA synthesis can be rescued with exogenous expression of *myc*. While Src is activated by bombesin treatment of Swiss 3T3 cells (Rodriguez-Fernandez & Rozengurt 1996), Src does not appear to be a critical player in bombesin-induced DNA synthesis because the Src-neutralizing antibodies were ineffective in blocking this event.

Src appears to be specifically involved in *myc* activation in PDGFβ receptor pathways; however, other receptors that activate Src may utilize this kinase for activation of distinct cellular pathways known to be involved in the induction of DNA synthesis. For example, v-Src activates MAP kinase and PI 3-K, signaling molecules known to participate in receptor-induced cell proliferation. The evidence implicating Src kinases in the phosphorylation of a few representative signaling molecules that are involved in receptor-induced DNA synthesis is discussed below.

Shc Tyrosine phosphorylation of Shc stimulates the Ras-MAP kinase pathway, which is critically involved in proliferation induced by v-Src and many proliferative receptors. Shc phosphorylation is detected following engagement of growth factor receptors, immune response receptors, GPCRs, cytokines, integrins, and some stress pathways. Microinjection of anti-Shc antibody inhibited DNA synthesis induced by insulin, IGF-1, and EGF, but not by serum (Sasaoka et al 1994). The induction of Shc phosphorylation by stress inducers and GPCRs may be mediated by RPTKs since these kinases are activated during

these responses, and dominant-negative, kinase-inactive EGFR can block activation of the MAP kinase pathway by GPCR agonists (Miller et al 1994, Coffer et al 1995, Daub et al 1996). Are Src family kinases involved in Shc phosphorylation by any of these receptors? Because Shc can directly interact with most receptor tyrosine kinases, it is unlikely that Src is involved in phosphorylation of Shc and activation of the Ras-MAP kinase pathway by these receptors. However, Src may be involved directly or indirectly in Shc phosphorylation by the other receptors. Src family kinases coprecipitate with Shc after stimulation of certain receptor pathways (Ptasznik et al 1995, Luttrell et al 1996), suggesting that Src kinases may directly phosphorylate Shc. Integrin-mediated Shc phosphorylation in certain cell types was recently shown to be independent of the α or β receptor cytoplasmic tails, and dependent on an interaction with another transmembrane protein, possibly caveolin (Wary et al 1996). Caveolin has been shown to interact with Src, thus this kinase or related PTKs could be involved in integrin-mediated Shc phosphorylation (Li et al 1996b). As described above, activation of Shc and EGFR tyrosine phosphorylation through $G_{\beta\gamma}$ subunits in COS7 cells is dependent upon the c-Src protein tyrosine kinase, and $G_{\beta\gamma}$ expression leads to the formation of Shc-Src and Shc-EGFR complexes (Luttrell et al 1996, 1997). These results suggest that $G_{\beta\gamma}$ activation of Src PTKs is essential for Shc phosphorylation, most likely through recruitment of Shc to the EGFR via interaction of the Shc SH2 or PTB domain with Src-dependent phosphorylation sites on the EGFR.

p85:p110 PI 3-K This PI 3-K isozyme is a heterodimeric enzyme consisting of a p85 regulatory subunit and a p110 catalytic subunit, which phosphorylates the D3 position of phosphatidylinositol (Carpenter & Cantley 1996). It is activated by v-Src, RPTKs, class I cytokines, G protein–coupled receptors, and other receptors. Activation of this isoform is mediated through interaction with the SH2 and SH3 domains of the p85 regulatory subunit, as well as through Ras interactions with p110. Recently, the oncogene of an avian retrovirus has been shown to encode a PI 3-K catalytic domain, indicating that under certain conditions, this enzyme is sufficient for constitutive stimulation of DNA synthesis (Chang et al 1997).

The role of PI 3-K in the induction of DNA synthesis varies in different cell types and for different receptors. For example, p85:p110 PI 3-K has been shown to be required for PDGF, but not CSF-1 (which does not induce D3 polyphosphatidylinositol accumulation), induced DNA synthesis (Roche et al 1995a). As with Shc, most RPTKs contain binding sites for p85 and do not appear to be dependent on Src for activation of this lipid kinase. However, the Src kinases activated by immune response receptors can be coprecipitated with p85, suggesting that Src may contribute to p85 phosphorylation (Augustine et al

1991, Yamanashi et al 1992, Pleiman et al 1993). GPCR activation of the MAPK pathway is inhibited by the PI 3-K inhibitor wortmannin, at a step upstream of tyrosine phosphorylation of Shc, indicating that PI 3-K (possibly a different isoform, γPI 3-K) is required for activation of the PTK that phosphorylates Shc (Hawes et al 1996). Because Shc phosphorylation by $G_{\beta\gamma}$ requires Src (Luttrell et al 1996), PI 3-K may be linked with the Src pathway in this system.

Growth factor receptors As described above, v-Src has been shown to phosphorylate several growth factor receptors (Wasilenko et al 1991, Peterson et al 1994, Stover et al 1995). These studies raise the possibility that c-Src is involved in RPTK activation by other receptor pathways. For example, thrombin-induced IGF-1R phosphorylation, ET-1-, LPA-, and thrombin-induced EGFR phosphorylation, and angiotensin II-induced PDGF-R phosphorylation could all involve c-Src. This possibility is supported by the evidence that c-Src is activated by all these agonists and that c-Src is required for $G_{\beta\gamma}$-induced activation of EGFR phosphorylation (Rao et al 1995, Linseman et al 1995, Daub et al 1996, van Biesen et al 1996, Luttrell et al 1997). Thus c-Src-mediated RPTK activation may be involved in the mitogenicity of some agonists that do not directly interact with receptor PTKs.

All these studies indicate that Src may participate in receptor pathways leading to the stimulation of DNA synthesis through a variety of mechanisms including, but not limited to, phosphorylation of Shc, leading to activation of MAP kinase pathway; activation of PI 3-K; and activation and/or phosphorylation of RPTKs.

G2/M TRANSITION Src is also involved in regulating a second stage of the cell cycle involving the G2/M phase transitions. The first hint that Src regulates events taking place during the G2/M phase of the cell cycle came from studies in *Xenopus* showing that v-Src accelerates meiotic maturation. However, it was not established whether this effect resulted from activation of the Ras/MAP kinase pathway or from other effector pathways operative during meiosis (Spivack et al 1984, Spivack & Maller 1985). More definitive evidence for a role for c-Src in somatic cell mitosis has been obtained from studies of Src in cells arrested during mitosis using the metaphase inhibitor, nocodozole, or in cells microinjected with Src inhibitory antibodies (Shalloway & Shenoy 1991, Fumagalli et al 1994, Taylor & Shalloway 1996). Src activity is elevated several-fold in metaphase-arrested cells, and this increase in activity correlates with Src phosphorylation by Cdc2 kinase at several serine and threonine residues in the N-terminal unique region. These and other studies suggest that phosphorylation by Cdc2 initiates a process leading to Src activation during mitosis. Roche and coworkers have further shown that Src inhibitory antibodies microinjected into G2 phase mouse fibroblasts blocked subsequent cell division prior to nuclear envelope breakdown (Roche et al 1995a). Src activity was required throughout G1 phase for induction of DNA synthesis. These results suggest that Src is involved in a function critical for progression from G2 to cell division. This function appears to be redundant with those of Fyn and Yes, which are also activated during mitosis, because an antibody that recognizes Src alone did not prevent cell division—only the cross-inhibitory anti-COOH peptide antibodies were effective.

One mitosis-specific v-Src substrate that could mediate Src effects during mitosis is $p68^{sam}$ (G Wong et al 1992, Taylor & Shalloway 1994, Courtneidge & Fumagalli 1994). This protein binds to Src via SH3 and SH2 domain interactions and shares significant homology with gld-1, Grp33 and shaking (Weng et al 1994, Taylor & Shalloway 1994, Jones & Schedl 1995, Ebersole et al 1996). All four proteins contain a KH RNA binding motif, which is located in the middle of the 170 amino acid homology region (referred to as GSG domain). HnRNP K, which contains three KH domains, also binds to the Src SH3 domain through a proline-rich motif similar to those in $p68^{sam}$. The gld-1 protein from *Caenorhabditis elegans* plays a negative regulatory role in mitotic activity during pachytene of oocyte meiotic prophase (Francis et al 1995a) and a non-essential role in negatively regulating proliferation of premeiotic germ cells (Francis et al 1995b). Loss of gld-1 function results in exit from pachytene, return to the mitotic cycle, and the development of tumors. Mutations in the KH domain show loss of function phenotypes, suggesting that this domain is required for tumor suppressor activity. It is tempting to speculate that $p68^{sam}$, like gld-1, negatively regulates mitotic progression. Tyrosine phosphorylation of $p68^{sam}$ may inhibit its function and relieve this negative control. However, there are notable differences between $p68^{sam}$ and gld-1 that raise doubts about whether these proteins share identical functional activities (no SH3 binding motifs in gld-1, no homology outside the GSG domain, nuclear localization of gld-1 not detected). Because the KH domain is involved in RNA and single-stranded DNA binding, the function of these KH motif proteins may be involved in RNA- or DNA-dependent binding events. $p68^{sam}$ has been shown to bind to RNA, and this binding is lost following tyrosine phosphorylation by Src (G Wong et al 1992, Taylor & Shalloway 1994, Wang et al 1995). Recently, HnRNP K was shown to have strand-specific single-stranded DNA binding activity and to act as a transcriptional activator, raising the possibility that other KH proteins also regulate transcription (Wang et al 1995, Michelotti et al 1995, 1996, Tomonaga & Levens 1996, Lee et al 1996).

It is possible that Src may regulate G2/M effectors other than Sam68, thus affecting alterations in the cytoskeleton or adhesion that take place during mitosis. Alternatively, Src kinases may be involved in activation of the Ras-MAP kinase pathway during mitosis. Raf has been shown to be activated during

mitosis (Laird et al 1995, Pathan et al 1996). In T cells, Raf binds to a mitosis-specific form of Lck that migrates with a retarded electrophoretic mobility. Mitotic activation of Raf was not observed in the Lck-deficient JCaM1.6 cell line, suggesting that Lck is involved in this cell-cycle specific activation of Raf (Pathan et al 1996). The Mos-MAPK pathway has been implicated in various aspects of mitotic and meiotic activities in *Xenopus* and mouse oocytes, at least partially related to spindle formation and polar body degradation (Minshull et al 1994, Gotoh & Nishida 1995, Choi et al 1996).

Apoptosis

The viability of vertebrate cells depends on signals transduced by survival factors that suppress apoptosis. Growth factors and cytokines, as well as extracellular matrix and cell-cell interactions, provide these signals that determine the fate of a cell to either survive (and possibly proliferate) or to undergo apoptosis. Signals transduced through a single receptor, e.g. the TCR or sIgM, can promote either apoptosis or cell proliferation depending on the way in which the cell is programmed to respond to receptor-proximal activation events.

Expression of v-Src has been shown to rescue several cell types from apoptotic death induced by either removal of cytokines, irradiation, chemotherapeutic drugs, or disruption of cellular interactions with extracellular matrix proteins (anoikis) (Anderson et al 1990, McCubrey et al 1993, Frisch & Francis 1994, Canman et al 1995, Basu & Cline 1995). These results suggest that constitutively activated Src can mimic the effects of cytokine receptors, integrins, and other receptor pathways that protect cells from certain programs of apoptosis. Studies in hematopoietic cells have provided evidence that the cellular homologues of Src kinases are involved in either protection from apoptosis or the induction of apoptosis.

TCR induction of cell death through the Fas pathway appears to be mediated through transcriptional induction of Fas ligand. Lck is required for induction of Fas ligand expression; however, the apoptotic pathway activated following Fas ligand binding to its receptor is independent of Lck (based on evidence that Lck-deficient T cells undergo Fas-induced cell death with efficacy similar to that of Lck-positive T cells) (Schraven & Peter 1995, Oyaizu et al 1995, Latinis & Koretzky 1996, Gonzalez-Garcia et al 1997). However, *fyn*-deficient mice are less sensitive to killing by anti-Fas antibody and Fas-ligand cytotoxic T cells, which suggests that Fyn may play an important role in Fas signal transduction (Atkinson et al 1996). sIgM-induced apoptosis is inhibited by antisense Blk in CH31 B-lymphoma cells (Yao & Scott 1993). Lyn and Fgr appear to prevent apoptosis during retinoic acid-induced granulocytic differentiation of HL-60 cells (i.e. *lyn* or *fgr* anti-sense oligodeoxynucleotides undergo premature apoptosis) (Katagiri et al 1996). In contrast, Lyn anti-sense oligonucleotides reverse the cell survival advantage provided to neutrophils by GM-CSF (Wei et al 1996). In addition, cross-linking of CD4 or stimulation by HIV-interaction with CD4 can lead to apoptosis (Corbeil et al 1996). This response requires the CD4 Cys-X-X-Cys, which is involved in Lck interaction. Introduction of Lck into Lck-deficient CD4+ T cells greatly increased HIV-induced apoptosis and syncytium formation. The apoptotic response did not require the kinase activity of Lck, suggesting that Lck may function as an adaptor in coupling with apoptotic signals.

These studies in hematopoietic cells suggest that Src kinases may play a role in regulating apoptosis; however, the precise mechanisms whereby Src exerts an effect in these cells remain to be established. The role of Src kinases in some of these systems could be indirect, involving coordination of initial events that trigger receptor-proximal signaling events (e.g. Lck phosphoryation of ITAMs in the TCR to initiate T cell responses). Alternatively, Src kinases could affect apoptotic responses through stimulation of pathways leading to MAP kinases or PI 3-K activation. Current studies indicate that the fate of a cell to either survive (and possibly proliferate) or to undergo apoptosis is dependent on the strength and balance of signals leading to activation of the various MAP kinase pathways (Erks, JNK/Sap-1s/p38Sap2s) as well PI 3-K (Yao & Cooper 1995, Xia et al 1995, Cuvillier et al 1996, Kauffmann-zeh et al 1996, Verheij et al 1996, Gardner & Johnson 1996, Johnson et al 1996, Park et al 1996a, Frisch et al 1996, Graves et al 1996, Minshall et al 1996, Wilson et al 1996, Kulik et al 1997). Thus to the extent to which Src kinases can regulate the activation of Erks and PI 3-K, these PTKs may participate in cellular pathways that regulate cell survival. Further studies are required to define the precise steps leading to or preventing apoptosis that are controlled by these kinases.

Differentiation

Expression of v-Src in immature cells can induce dramatic changes in the differentiation program of the cells. In most cell types, v-Src expression blocks cell differentiation. For example, infection of avian myoblasts, retinoblasts, or chondroblasts with RSV maintains these cells in a proliferative state and blocks differentiation into myotubes, neuroretinal cells, epidermal cells, or chondrocytes, respectively (Muto et al 1977, Yoshimura et al 1981, Crisanti-Combes et al 1982, Alema & Tato 1987). In contrast, introduction of v-Src into PC12 cells or immature sympathetic neurons induces neurite outgrowth and terminal differentiation into neuron-like cells (Alema et al 1985, Haltmeier & Rohrer 1990, Hecker et al 1991). As discussed previously, these dramatic alterations in cell differentiation induced by v-Src do not imply that cellular Src mediates these effects when activated under natural conditions. Although Src kinases may be involved in some of these responses under natural conditions, it is likely that

v-Src, as an unregulated, promiscuous PTK, mimics the cellular activities of other protein tyrosine kinases through phosphorylation of common substrates, and that the nature of the cellular responses to v-Src is dependent on how the cell is programmed to respond to the signals activated by high level, sustained phosphorlyation of these substrates.

A comparison of the biochemical responses of v-Src and NGF in PC12 cells indicated that many of the changes induced by v-Src mimic those of NGF, including phosphorylation of cellular proteins, induction of gene transcription, and priming to NGF (Rausch et al 1989, Thomas et al 1991). Together, these results suggest that v-Src's ability to function as an inducing agent is a consequence of its ability to mimic critical aspects of the NGF differentiation program. However, c-Src would appear to be a critical component of NGF's actions because microinjection of anti-Src monoclonal antibodies blocks NGF-induced neurite outgrowth (Kremer et al 1991). Thus as in the example of proliferative responses to PDGF, CSF-1, and EGF receptors, c-Src appears to contribute to the cellular responses mediated by these RPTKs, but the activation of Src by these receptors would not appear to be sufficient for induction of either cell proliferation or differentiation.

The ability of v-Src to block differentiation of immature cells raises the question of whether the block in differentiation is indirectly a consequence of the stimulation of cell proliferation or whether it can alter transcription of differentiation-specific genes independently of cell proliferation. In myoblasts, this issue has been addressed by activating a temperature-sensitive mutant of v-Src following myotube differentiation. Expression of v-Src led to inhibition of transcription of many muscle-specific genes in the differentiated myotubes in the absence of an induction of cell proliferation, which suggests that Src is able to inhibit the myogenic transcriptional program via a more direct mechanism (Schneider & Olson 1988, Falcone et al 1991). v-Src also induces transcription of many differentiation-specific genes in fibroblasts and other cell types, e.g. αD globin and keratin 18 (Pankov et al 1994, Itoh-Lindstrom & Leffak 1994). These results indicate that activated variants of Src can feed into transcriptional control pathways that regulate cell differentiation, thus raising the possibility that cellular homologues of these genes can participate in the pathways as well.

The role of Src kinases in differentiation of hematopoietic cells has been examined very thoroughly through studies of mice lacking single kinases or combinations of different kinases. For an extensive description of these studies and a discussion of the IRR and MHC receptors, see Lowell & Soriano (1996).

Gene Transcription

Many of the biological activities discussed in this section involve changes in gene transcription. Studies of v-Src in fibroblasts have defined many cellular genes whose transcription is positively or negatively regulated by v-Src expression (Bedard et al 1989, Jahner & Hunter 1991, Qureshi et al 1991, Qureshi et al 1992, Frankfort & Gelman 1995, Scholz et al 1996). v-Src transcriptional activation involves multiple transcriptional control elements, including NF-κB, ATF/CRE, AP-1, CArG, and SIE sites (Dehbi et al 1992, Eicher et al 1994, Xie et al 1994, Simonson et al 1996, Cao et al 1996). The best example of a receptor-mediated transcriptional event regulated by c-Src would be the control of *myc* transcription by the PDGFβ receptors (Barone & Courtneidge 1995). Inhibition of c-Src with an inhibitory antibody blocked activation of *myc* transcription. Endothelin-induced *fos* induction also appears to be regulated by Src because expression of a kinase-inactive Src mutant blocks the endothelin induction of this gene (Simonson et al 1996).

IN VIVO BIOLOGICAL ACTIVITIES OF SRC KINASES

It is clear from the discussion above that Src PTKs couple with a plethora of receptors, engage unique and common sets of targets, and participate in the regulation of diverse biological responses. Most of the studies discussed involved analysis of immortalized cell lines or isolated primary cells. Examining the functions of these kinases in whole organisms is important for understanding how these molecular and cellular activities relate to both normal and disease-associated physiological events that involve Src PTKs.

Understanding the normal physiological role of Src PTKs has been aided by the advent of gene targeting and embryonic stem cell technology in the mouse. In addition, studies in *Drosophila* have aided in understanding Src PTK functions in vivo. Targeted disruptions of all known mammalian Src PTK genes have been generated in mice. The phenotypes of these mutant mice range from no overt defects to very distinct abnormalities in specific cell types, tissues, or physiological responses. For a detailed summary of these phenotypes, see reviews by Lowell & Soriano 1996 and Brown & Cooper 1996. The section below focuses on how the phenotypic effects of *src*, *fyn*, and *lck* disruption in mice relate to the roles of these kinases in the receptor pathways covered in this review.

Effects of Src Disruption in Mice

As indicated above, disruption of Src function in the mouse results in osteopetrosis, a bone remodeling defect (Soriano et al 1991). A more severe form of osteopetrosis is seen in *hck/src* double disruption mutants (Lowell et al 1996b). While the osteopetrotic phenotype of *src-/-* mice was an unexpected finding (given the predicted role of Src in cell proliferation and the absence of evidence suggesting a role for Src in bone physiology), subsequent studies revealed that

Src is expressed at very high levels in osteoclasts, cells involved in bone remodeling (Horne et al 1992). Hck is also present at high levels in osteoclasts (Lowell et al 1996b). Bone remodeling involves a dynamic balance between bone resorption and bone deposition. Osteopetrosis results when this balance is perturbed either through decreased resorption or excess deposition. In *src*-deficient mice, osteopetrosis is the result of a defect in bone resorption mediated by osteoclasts. Osteoclasts are present in the *src*-deficient mice (in fact, in excess); however, these cells display severe defects in bone resorption in vivo and in vitro (Boyce et al 1992, Lowe et al 1993). Bone resorption by osteoclasts involves the formation of a ruffled border, which secretes proteases, hydrogen ions, and other bone hydrolyzing agents at the site of osteoclast adhesion to bone. Formation of the ruffled border is defective in *src-/-* osteoclasts (Boyce et al 1992).

The molecular basis for this phenotype is not understood; however, the osteoclast resorption defect could involve alterations in at least two different receptor pathways. Attachment of the osteoclast to the bone is thought to be mediated by an interaction between the ECM molecule, osteopontin (OPN) and its receptor, the $\alpha_v\beta_3$ integrin. Src has been found to co-immunoprecipitate with the $\alpha_v\beta_3$ integrin and is also activated after OPN treatment (Rolnick et al 1992, Hruska et al 1995, Chellaiah et al 1996). Thus loss of Src could affect OPN or other matrix signaling responses necessary for the final maturation of osteoclasts following adhesion to bone. Interestingly, analysis of OPN expression in osteoclasts from Src-deficient mice shows a decrease in the level of OPN protein (Chackalaparampil et al 1996). Thus Src function may affect both ligand production and signaling by this adhesion receptor.

Alternatively, RPTK pathways may be impaired by loss of Src. Osteoclasts express several RPTKs, including the CSF-1 receptor and two HGF family members (Grano et al 1996, Kurihara et al 1996). Loss of the CSF-1 gene in mice causes osteopetrosis. However, this phenotype is the result of a defect in osteoclast differentiation, rather than osteoclast function (Yoshida et al 1990). Because Src PTKs can associate with the CSF-1 receptor and are activated after CSF-1 treatment (Courtneidge et al 1993), it is possible that Src is required for specific functions of the CSF-1 receptor that are necessary for resorption activity in mature osteoclasts but are dispensable during osteoclast maturation. CSF-1 treatment of osteoclasts induces cell spreading, an induction of total cell phosphotyrosine, and activation of Src (Insogna et al 1997). Src appears to be important for CSF-1 signaling in osteoclasts because *src-/-* osteoclasts do not spread in response to CSF-1. Thus defects in CSF-1 signaling could contribute to the osteoclast defect in *src-/-* mice.

A second family of RPTKs, the HGF/SF receptor family, may also play a role in osteoclast function. Osteoclasts express at least two HGF receptor family members, STK and Met (Grano et al 1996, Kurihara et al 1996). STK is a receptor for macrophage stimulating protein (MSP). MSP treatment of osteoclast-like cells (derived by culturing of murine bone marrow in the presence of 1,25-dihydroxyvitamin D3 and interleukin-3) results in formation of a ruffled border and redistribution of Src to these borders. This correlates with an increase in bone resorption (Kurihara et al 1996). The ligand (HGF) for the closely related family member Met is secreted by osteoclasts. HGF treatment of osteoclasts increases Src kinase activity and alters osteoclast cell shape (Grano et al 1996). Thus defects in these RPTK pathways owing to the loss of Src could play a role in the osteopetrotic phenotype of *src-/-* mice. Analysis of whether loss of Src affects ligand secretion and/or signaling by these receptors should lead to insight into the molecular and cellular basis for the defects observed in Src-deficient mice.

Osteoclasts from *src-/-* mice display decreased levels of total cell phosphotyrosine-containing proteins relative the *src+/+* osteoclasts (P Schwartzberg, HE Varmus, unpublished results). Because several downstream targets of Src such as FAK, tensin, cortactin, and PI 3-K are likely involved in osteoclast function, decreased tyrosine phosphorylation of some of these proteins could be involved in the osteoclast defect (Berry et al 1994, Hiura et al 1995, Hruska et al 1995) . Cbl tyrosine phosphorylation is impaired in *src-/-* osteoclasts and in wild-type osteoclasts treated with *src* anti-sense oligonucleotides (Tanaka et al 1996). Although tyrosine phosphorylation of other proteins has not been carefully examined in *src-/-* osteoclasts, cortactin, tensin, and FAK are found in the ruffled borders of osteoclasts, lacking *src-/-* osteoclasts (Berry et al 1994, Hiura et al 1995). In addition, PI 3-K is associated with the $\alpha_v\beta_3$ integrin and there is an increase in D3 phosphatidylinositols in OPN-treated osteoclasts (Hruska et al 1995).

Although some of these proteins may be direct substrates of Src, transgenic studies suggest that Src has a kinase-independent function in osteoclasts. Expression of wild-type, autophosphorylation-deficient, or a kinase-inactive transgene in *src-/-* mice can rescue the osteopetrotic defect (P Schwartzberg, L Xing, B Boyce & HE Varmus, unpublished results). Analysis of tyrosine phosphorylation indicates that the expression of kinase-inactive Src restores tyrosine phosphorylation, although there may be some differences in the overall profile of proteins phosphorylated. These results suggest that direct phosphorylation by Src of a subset of proteins is unlikely to be required for osteoclast function. Instead, Src may be important for regulating the localization of certain proteins or stabilizing signaling complexes that are necessary for formation of the ruffled border and bone resorption. Consistent with this hypothesis, Cbl, a scaffolding protein that binds to multiple signaling proteins, is not localized properly in Src-deficient osteoclasts (Tanaka et al 1996). In addition, in avian

osteoclasts, Src appears to associate with microtubules, and this association is not dependent upon tyrosine phosphorylation (Abu-Amer et al 1997). Because the microtubule network is important for transport of proteins to the ruffled border, it is possible that loss of Src could affect microtubule-based protein trafficking in osteoclasts.

Whether impairment in integrin, RPTK, or other receptor pathways is responsible for the osteopetrotic phenotype in Src-deficient mice is unclear. However, these studies, together with previous work on Src PTKs, provide some testable hypotheses for understanding the molecular basis for Src function in vivo.

Effects of Fyn and Lck Disruption on T Cell Development in Mice

The role of Fyn and Lck in T cell development and activation has been examined through analysis of mice that either overexpress mutant or wild-type forms of these kinases or are deficient in expression of these proteins due to disruption of the endogenous genes. Twentyfold overexpression of FynT in mice leads to enhanced TCR responses in thymocytes (Cooke et al 1991). In contrast, overexpression of a kinase-inactive form of FynT significantly inhibited TCR-mediated activation in otherwise normal thymocytes. Thymocytes from mice lacking either FynT or FynT plus the ubiquitously expressed form of Fyn (FynB) show significant reductions in calcium and proliferative responses to stimulation through the TCR. However, peripheral T cells re-acquired significant signaling responses (≈50% of the wild-type response), and splenic T cells displayed only a partial or undetectable response to alloantigen (Appleby et al 1992, Stein et al 1992). These results suggest that the functions of Fyn in TCR stimulation are redundant in peripheral T cells, but to a much less significant extent in thymocytes.

Although disruption of Fyn does not affect maturation of thymocytes, Lck disruption results in a significant reduction in the number of CD4+/CD8+ thymocytes, thus Lck may play a critical role in early thymocyte maturation (Molina et al 1992, Lewin et al 1993, Penninger et al 1993). However, small populations of CD4+/CD8+ thymocytes can develop into mature $\alpha\beta$ T cells that are partially responsive to TCR-mediated stimulation (Molina et al 1992, Penninger et al 1993, Kawai et al 1995). Peripheral T cells from mice lacking Lck show severe defects in TCR signaling by antigen, however the same cells could be stimulated by anti-CD3, suggesting that Fyn is able to compensate for the absence of Lck when a large population of TCR/CD3 is engaged under receptor cross-linking conditions. The functional redundancy of Lck and Fyn in T cell signaling and maturation was further supported by the demonstration that disruption of both the Fyn and Lck genes completely arrests $\alpha\beta$ T cell development at the CD4-/CD8-stage (van Oers et al 1996b, Groves et al 1996). In addition, expression of an activated FynT transgene can restore production of mature CD4+/CD8+ double-positive thymocytes and $\alpha\beta$ cells and improve representation of CD4+ and CD8+ single-positive cells in Lck-deficient mice (Groves et al 1996). These results suggest that Lck is the primary Src-related kinase involved in thymocyte maturation but that Fyn can partially compensate for Lck, albeit much less effectively. This conclusion is supported by the evidence that the constitutive ζ phosphorylation found in fresh thymocytes is severely reduced in thymocytes from Lck-/- mice (van Oers et al 1996a).

Is the prominent role of Lck in early T cell development a consequence of a unique function of this kinase or of its specific expression in these cells? Although differences in the levels of expression could contribute to the different dependencies on Fyn and Lck, several lines of evidence suggest that the functional activities of these kinases are distinct (Olszowy et al 1995). As discussed above, kinase-inactive Lck disrupts thymocyte development, whereas kinase-inactive Fyn does not, and expression of active Lck causes thymic tumors, whereas Fyn only induces hyperstimulatable T cell responses (Anderson & Perlmutter 1995). Fyn can be coprecipitated or cocapped with the TCR, whereas Lck is not detectable under similar conditions (Samelson et al 1990, 1992, Gassman et al 1992), and Lck couples with CD4 and CD8 whereas Fyn does not. In addition, differential roles of FynT and FynB were inferred from the ability of activated variants of FynT, but not FynB, to cause an increase in TCR-stimulated IL-2 production (despite similar hyper-responsiveness as measured by induction of tyrosine phosphorylation) (Davidson et al 1992). FynT was also shown to be more efficient in promoting antigen receptor-triggered calcium fluxes, and calcium ionophores partially rescue FynB's inability to enhance antigen-mediated lymphokine secretion (Davidson et al 1994). The distinct catalytic domain sequences of FynT, not the distinct SH2 sequences, are responsible for the improved ability to augment antigen responsiveness and calcium mobilization. Thus the unique structural features of FynT found within the N-proximal region of the cataltyic domain are responsible for the specific effects of this Fyn isoform in T cell signaling. It will be of interest to determine whether this difference affects substrate specificity of Fyn.

Effects of Fyn Disruption on Neural Functions

Loss of Fyn in mice also causes defects in neural functions, although the molecular basis for the neurological impairments is less well understood. Loss of Fyn has been associated with abnormalities in the hippocampus, defects in long-term potentiation (LTP), reduced myelination, increased fear response, and decreases in the rate of amygdala kindling (Grant et al 1992, Umemori et al 1994, Cain et al 1995). Although little is known about the molecular basis for amygdala kindling, some molecular events involved in LTP and myelination have been identified.

HIPPOCAMPUS/LTP Disruption of the *fyn* gene results in abnormalities in hippocampal histomorphology manifested by an increase in granular cells in the dentate gyrus and pyramidal cells in the CA3 region (Grant et al 1992). Whether loss of Fyn increases the rate of cell proliferation, alters cell fate decisions, or inhibits apoptosis is unclear. Since Fyn has been linked to apoptosis in T-cells (see IRR section), it is possible that decreased apoptosis may contribute to the hippocampal defects (Atkinson et al 1996). In order to understand how loss of Fyn affects hippocampal development it is critical to determine what factors are important for generation and maintenance of these cell populations.

The abnormalities in the hippocampal architecture could contribute to the defects in LTP, which are also observed in *fyn-/-* mice. The hippocampus is important for memory and learning, and these processes have been linked to LTP. The impaired LTP in *fyn-/-* mice has been detected in the synapses formed by the CA3 pyramidal cells and the CA1 neurons of the hippocampus. Thus it is possible that the increase in pyramidal cell number is responsible for this defect as well (Grant et al 1992). However, the hippocampal architectural defects can be distinguished from the LTP defect because expression of a *fyn* transgene in the forebrain can rescue the LTP defect but does not correct the altered hippocampal morphology (Lowell & Soriano 1996). Therefore, a second potential explanation for the LTP phenotype observed in *fyn-/-* mice is a defect in the neurotransmitter signaling pathway activated at the synapse. The major neurotransmitter found at the CA3/CA1 synapse is L-glutamate, which binds to NMDA and non-NMDA receptors on the post-synaptic CA1 neurons (Collingridge et al 1983). Activation of the NMDA receptors initiates a cascade of signaling events, including changes in intracellular calcium and activation of serine/threonine kinases and tyrosine kinases (Grant & Silva 1994). Recently, Src was shown to interact directly with the NMDA receptor via its unique domain, and antibodies to Src are able to decrease channel gating (Yu et al 1997). Both Src and Fyn phosphorylate the NMDA receptor and this phosphorylation is likely important for receptor function (Suzuki & Okumura Noji 1995, Yu et al 1997). Because there are no obvious defects in LTP in neurons of Src-deficient mice, these results suggest that Src phosphorylation and association with the receptor is not important for LTP (Grant et al 1992). It is possible that events downstream of the NMDA receptor are more severely affected in Fyn-deficient mice. A decrease in FAK tyrosine phosphorylation has been observed in the hippocampus of Fyn-deficient mice, but whether FAK plays any role in NMDA signaling is uncertain (Grant et al 1995). Defects in LTP have also been observed when NCAM function or Ca^{2+}/calmodulin-dependent kinase II (CamKII) function is blocked (Grant & Silva 1994, Mayford et al 1995, Bach et al 1995, Ronn et al 1995, Glazewski et al 1996). Both molecules have been proposed to function downstream of the NMDA receptor. Given that neurons from Fyn-deficient mice are impaired in their ability to extend processes on NCAM-expressing fibroblasts, it is possible that analogous defects in the hippocampus may contribute to LTP impairment (Beggs et al 1994). Similarly, although there is no direct link between CamKII and Fyn, it is possible that loss of Fyn could be affecting CamKII signaling pathways. CamKII is activated by intracellular Ca^{2+} (Hanson & Schulman 1992). In endothelial cells, activation of CamKII-dependent signaling pathways precedes thrombin-induced tyrosine phosphorylation (Marsen et al 1995). As mentioned above, Ca^{2+}-induced differentiation of mouse keratinocytes results in Fyn activation (Calautti et al 1995). Thus loss of Fyn could affect CamKII signaling, which could contribute to the LTP defects. Determining how loss of Src and Fyn affects NMDA receptor tyrosine phosphorylation and NMDA signaling pathways should elucidate whether defects in this receptor signaling pathway are linked to the impairment of LTP in Fyn-deficient mice.

FEAR RESPONSE An increased fear response has also been observed in Fyn-deficient mice (Miyakawa et al 1996). This defect has been observed in mice carrying an in-frame insertion of a β-galactosidase (lacZ) gene downstream of sequences in Fyn encoding the SH3 domain. This results in the production of a protein composed of the Fyn unique and SH3 domains fused to lacZ. The fear response has not been measured in *fyn* null mice, so this defect could be specific to this partial mutation. In any case, these results suggest that there may be multiple behavioral abnormalities due to loss of Fyn.

MYELINATION DEFECTS In addition to the architectural abnormalities in the hippocampus and the impaired LTP, some Fyn-mutant mice also show reduced myelination (Umemori et al 1994). The defect was originally observed in the in-frame lacZ mice described above. Analysis of myelination has not been done in *fyn* null mice; thus it is difficult to predict whether this defect results from the loss of Fyn. It should be noted, however, that Fyn can associate with myelin-associated glycoprotein (MAG) (Jaramillo et al 1994). MAG is an adhesion molecule implicated in myelination. Cross-linking of MAG induces a rapid increase in total cell phosphotyrosine and also activates Fyn. Thus defects in myelination observed in these Fyn mutants may be explained by disruption of MAG signaling.

Effects of Disruption of Both Src and Fyn in Mice

Given the large number of receptors to which Src PTKs couple, it is somewhat surprising that mutations in Src PTKs are tolerated during embryogenesis; however, it is also clear that multiple Src PTKs are involved in each receptor pathway. Thus the lack of a phenotype in mice containing a single Src gene disruption likely is due to the functional redundancy in tissues where these

kinases are highly expressed and where multiple Src PTKs are activated by the same receptors. Consistent with this hypothesis, more severe defects are observed when mutations are made in multiple Src PTKs (Lowell & Soriano 1996). Mutations in more than one Src PTK may be necessary in order to reveal a requirement for these kinases in particular systems. The more severe T cell defect in *fyn-/-;lck-/-* mice and the osteoclast defect in *hck-/-;src-/-* mice are consistent with this hypothesis (van Oers et al 1996b, Lowell et al 1996b). *fyn-/-;src-/-* mice die perinatally (Stein et al 1994). Although the basis for the lethality is unclear, detailed analysis of these mice may reveal requirements for Src PTKs in additional receptor pathways in vivo and provide insight into the molecular basis for the phenotype.

Drosophila Src Kinases

One of the surprising findings from studies of *src-/-* mice was the absence of any neuronal phenotype (Lowell & Soriano 1996). As mentioned in the introduction, Src is expressed at high levels in brain, and there are two alternatively spliced, neuron-specific forms of Src, referred to as Src(+). Closely related alternatively spliced mRNAs of Src have also been identified in *Xenopus laevis*. There are two Src-related genes in *X. laevis*, and proteins encoded by alternatively spliced Src mRNAs have a 5-amino acid insert. In addition, expression of these isoforms is observed after neural induction but before differentiation (Collett & Steele 1993). Although the evolutionary conservation of the alternatively spliced form of Src suggests that it is important, no definitive role for Src(+) has been found. Mammalian cell culture studies, however, suggest that c-Src is required for neurite outgrowth in response to various differentiation agents (Kremer et al 1991). Although it is possible that Src or Src(+) are not important for neural function in vivo, it is likely that the role of this kinase is masked by the presence of at least three other Src PTKs. Consistent with this idea, studies in *Drosophila* suggest that Src may be important in the nervous system in this organism. Three Src-related genes have been identified in *Drosophila* (Simon et al 1985, Gregory et al 1987, Takahashi et al 1996). Dsrc29 is more closely related to the Tec family kinases (Gregory et al 1987). The remaining two Src-related molecules, Dsrc64 and Dsrc41, share homology with Src throughout their sequences (Simon et al 1985, Takahashi et al 1996). Overexpression of these Src homologues in *Drosophila* embryos results in distinct consequences (Kussick et al 1993, Takahashi et al 1996). Dsrc41 overexpression has no effect on *Drosophila* development, whereas overexpression of wild-type Dsrc64 in *Drosophila* embryos is lethal, and expression in specific neuronal precursor cells of the eye disrupts eye development (Kussick et al 1993, Takahashi et al 1996). Biochemical analysis of Dsrc64 reveals that it is phosphorylated on the negative regulatory tyrosine as well as on the positive regulatory autophosphorylation site, suggesting that this kinase may be activated during embryonic development, either through a receptor pathway or through incomplete phosphorylation by the *Drosophila* Csk homologue (Kussick et al 1993, Takahashi et al 1996). Thus similar to higher eukaryotic Src family kinases, these kinases may have slight differences in their regulatory mechanisms.

Expression of kinase-inactive Dsrc64 or Dsrc41 in certain neuronal precursors of the *Drosophila* eye disturbs normal eye development, which suggests that Src may play a role in neural development (Kussick et al 1993, Takahashi et al 1996). Although a similar phenotype has not been observed in flies that are null for Dsrc64 (see below), the presence of at least one other Src-related gene, Dsrc41, could account for the difference in phenotype.

Studies of flies expressing kinase-defective and -activated Dsrc41 in the neuronal precursor cells of the eye have also provided some clues to downstream pathways regulated by *Drosophila* Src (Takahashi et al 1996). Expression of an activated form of Dsrc41 (Dsrc41YF) results in an increase in the number of R7 photoreceptor cells. This phenotype is dependent on two receptor tyrosine kinases, Sevenless and the *Drosophila* EGF-R, which suggests that Dsrc41 interacts with RPTK pathways (Cagan 1993, Takahashi et al 1996). Defects in the formation of adherens junctions and organization of the actin cytoskeleton in preclusters are observed when the kinase-inactive mutant is expressed in precursors of R3 and R4. Specifically, there is a loss of actin fibers and cadherin localization to the cell-cell junctions between the R3 and R4 precursors. This phenotype is enhanced when there is a single dose reduction of the endogeneous Dsrc41. These results suggest that Dsrc41 may regulate the cytoskeleton and cell-cell interactions.

Further support for a role for Src PTKs in cytoskeletal organization comes from analysis of *src64* null flies (M Simon, personal communication). Generation of a mature oocyte involves coordinated changes in the cytoskeleton. Female flies homozygous for a null mutation in Dsrc64 have a partial infertility defect that may be linked to oocyte development. *Drosophila* ovaries are composed of egg chambers; within each chamber are 15 nurse cells and a single oocyte. The cytoplasm of these cells is connected by structures called ring canals. These structures allow transport of nutrients from the nurse cells to the oocyte, and in one of the latter stages of development of the oocyte, the transfer of the cytoplasm of the 15 nurse cells into the single oocyte. This transfer results in expansion of the oocyte and shrinking of the nurse cells (Cooley & Theurkauf 1994). Dsrc64-null females have smaller eggs, and the eggs that do not produce larvae are unfertilized. The decreased egg size likely results from incomplete transfer of the nurse cell cytoplasms to the oocyte. The defect in transfer could be due to alterations in the ring canal. Ring canals of Dsrc64 null

females are smaller and occasionally become detached. In addition, whereas the ring canals normally stain with phosphotyrosine antibodies, ring canals from Dsrc64 null females are devoid of phosphotyrosine epitopes (M Simon, personal communication).

The lack of fertilization may also be linked to the ring canals/nurse cells. The micropyle structure is important for sperm entry. This structure is derived from border cells that migrate from the anterior pole of the egg chamber between the nurse cells to the nurse cell-oocyte border (Lehman 1995). The presence of aberrant nurse cells could perturb border cell migration, thereby affecting formation of the micropyle structure. Using sensitized genetic screens, dominant mutations have been identified that enhance the phenotype of a Dsrc64 weak allele. Interestingly, one of these mutations occurs in the Dsrc29 gene, which is most closely related to the tec family kinase BTK (M Simon, personal communication).

Thus studies on the *Drosophila* Src PTKS suggest that, similar to their mammalian counterparts, *Drosophila* Src-related kinases can interact with other PTK families and regulate similar activities (adhesion/cytoskeleton).

SUMMARY

The experimental studies discussed above clearly indicate that Src kinases are versatile enzymes that play key roles in regulating many biological activities induced by numerous cellular receptors. A central question raised by the promiscuity of these kinases is how signals that activate Src kinases can mediate distinct biological events following activation by different receptors. The same question could be raised for many different receptor-activated signaling enzymes, e.g. Ras, PI 3-K, protein kinase A and PKC. Like Src kinases, these enzymes are activated by a broad spectrum of receptors and have been implicated in regulation of many biological events. Thus the question can be more broadly stated: How does each receptor elicit distinct cellular responses utilizing a similar set of signaling molecules? Current evidence indicates that there are several factors that affect the biological consequences of activation of a specific signaling protein.

1. Qualitative and quantitative aspects of protein expression: Each cell expresses a unique set of specific isoforms of each class of protein and different levels of expression of each protein. Further specificity can be derived from alternative splicing of transcripts to alter the activity or coupling of signaling proteins.

2. Cellular localization: Each receptor has evolved unique strategies to recruit specific sets of proteins to signaling complexes. Thus, any one particular

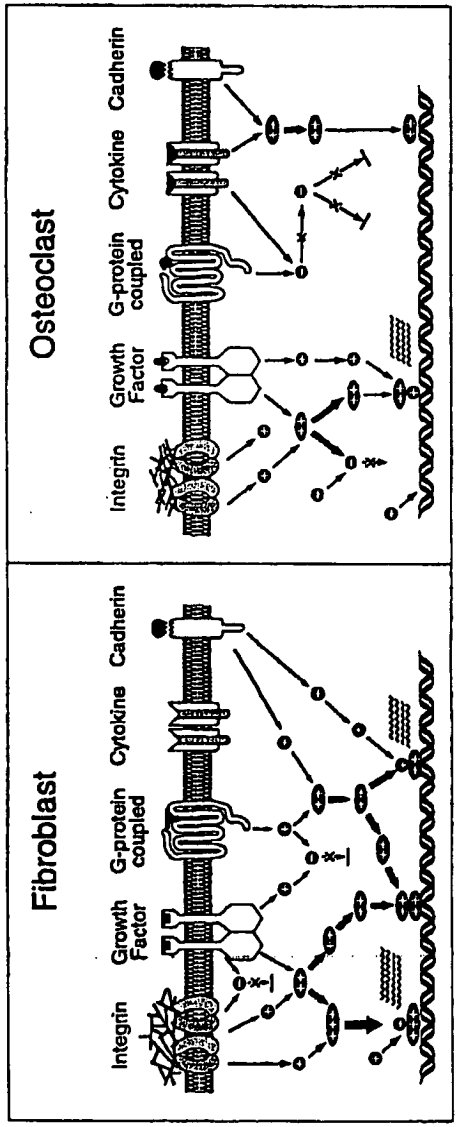

Figure 7 Combinatorial signal transduction.

enyzme may be in contact with, or proximal to, distinct effector molecules following activation by different receptors. Src PTKs and other signaling enzymes contain multiple binding domains allowing these proteins to couple with many other proteins that determine their subcellular compartmentalization.

3. Combinatorial effects: Because any one cell expresses multiple receptors that are in an activated or basal state under different conditions and because each of these receptors can cross-talk with other receptor signaling pathways through interactions with common downstream effectors, the strength of any one signal and its duration of action can be strongly influenced by synergistic or antagonistic influences from other receptor pathways.

Receptor signal transduction pathways are often compared to electrical circuitry networks, where the eventual outcome is dependent not only on the set of individual switches incorporated into the circuit board, but also on the combination of signals that are on or off at any one time. As illustrated in Figure 7, the factors described above all contribute to the combinatorial effects that allow Src to control bone resorption in an osteoclast and DNA synthesis in a fibroblast. The ultimate goal of research in this area is to be able to draw a complex circuitry scheme that explains how signals are processed in each cell. While reaching this goal may be far off, current research elucidating how proteins couple in distinct receptor pathways is laying the framework for future studies of how signals emanating from many different receptor pathways integrate with each other to control cell behavior.

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